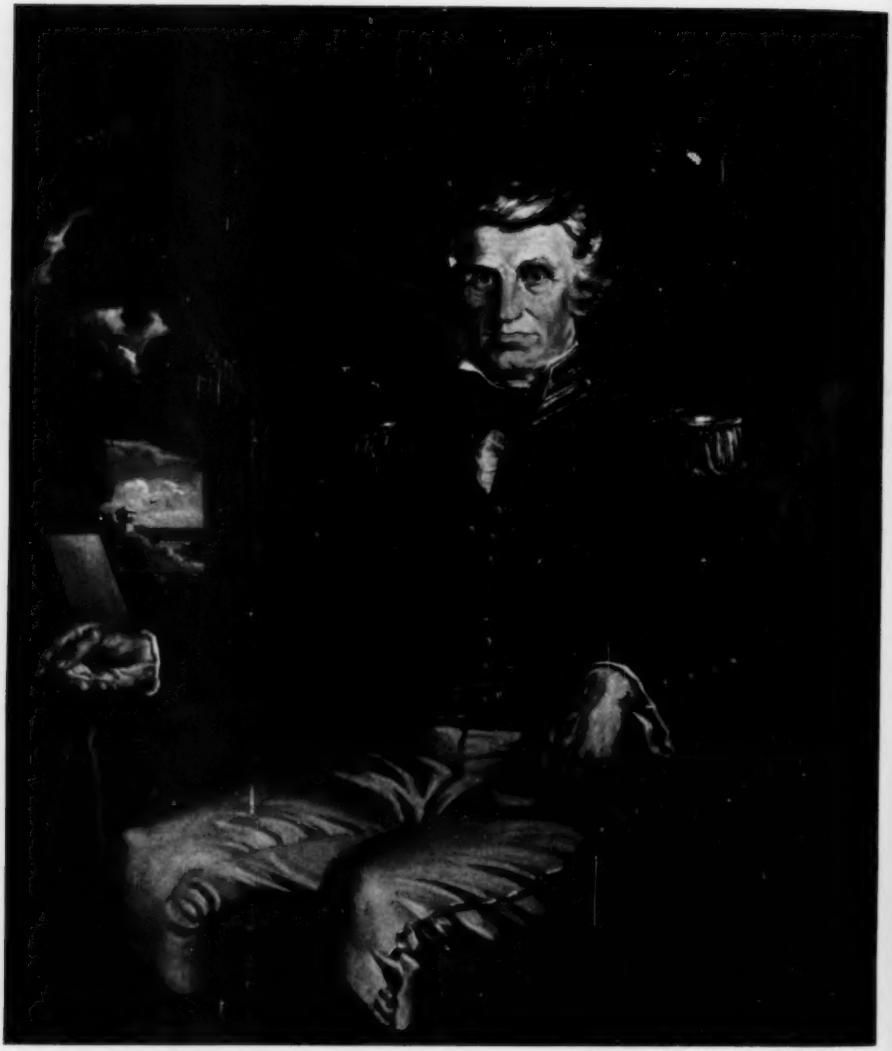
WILLIAM BEAUMONT

1785 – 1853



— Courtesy of Yale University Art Gallery

William Beaumont

Painted in 1943 by Deane Keller for the Beaumont Medical Club and now hung in their Room in the Yale Medical Library.

WILLIAM BEAUMONT

(November 21, 1785 – April 25, 1853)

The fame of William Beaumont is well-established in American scientific lore; the school-boy whose participation in the science of physiology is, at best, in the hazy future has heard in some detail of this energetic doctor who carried out experiments on a man with a hole in his stomach in a backwoods settlement in upper Michigan more than a hundred years ago. Similarly, the medical student is reminded of the endless possibilities for service and discovery which are open to the prepared mind, as exemplified in the career of Beaumont. The physiologist and biochemist are awed anew each time they read in Beaumont's own words the highly objective accounts of his observations of the behavior of the stomach of a human subject under a variety of experimental conditions, for the results of investigation in the field of gastric physiological chemistry during the intervening years have demonstrated the essential accuracy of his findings.

As is indicated in the foregoing, the story of Beaumont's experiments is well-known; it is of interest to speculate on the qualities in his character which might reasonably account for this remarkable career, once the opportunity for suitable expression presented itself. His ancestors, stalwart New Englanders, had a recorded history before him for 5 generations, the first American in the line settling in Saybrook, Connecticut, about 1640. The men seem to have acquired property and to have taken part in local politics, and when the Revolutionary War broke out Beaumont's father enlisted for several three-year periods. He returned to the family homestead in Lebanon, Connecticut, early in 1785 and married. William Beaumont was born there on November 21, 1785. He grew up in a home where civic and religious obligations were taken seri-

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ously and in addition he had the advantages of excellent local schooling. The new republic was developing a profitable foreign trade, domestic national politics were assuming a workable pattern, and the first suggestions of war with England were heard as Beaumont left home in the winter of 1806–1807. He had no desire to be a farmer but instead settled in Champlain, New York, where for three years he taught the village school. He entered into the social life of the town and, after deciding to become a doctor, read avidly in borrowed medical books. In 1810 he began the serious study of medicine with Dr. Benjamin Chandler in St. Albans, Vermont. His first-hand experience in anatomy consisted of the dissection of every amputated arm or leg he could secure; he urged autopsies at the death of his patients and from these, too, he learned anatomy. He secured his license to practice medicine in 1812 and very soon thereafter joined the U. S. Army at Plattsburgh.

Beaumont was deeply conscious of his debt to his preceptor, Dr. Chandler, with whom he acquired first-hand experience in the pratice of medicine. In this period, as throughout his life, he kept journals not only of his professional experiences but of his personal views as well. He early recognized the value of a good reputation and appears to have acquired testimonials regarding his character from all of his important personal and professional contacts. From the voluminous notes, letters and journals which he left, one gets the impression that the personal characteristics of scrupulous honesty, self-evaluation, and serious ambition were well-established in Beaumont's early maturity, and there is little doubt that these attributes played a significant part in his perception of the great opportunity later to be presented to him, as well as in his conduct toward it.

Beaumont took part in the War of 1812, seeing service as a military surgeon from February, 1813, to August, 1814, largely in the region around the mouth of the Niagara River and in Plattsburgh. Even before leaving Plattsburgh and throughout the campaign in 1813 he kept voluminous notes bearing on his medical experiences — the therapeutic and surgical treat-

ment employed and, wherever possible, the results obtained, all in the minutest detail. In 1815 he resigned from the Army, finding that the routine of military duty did not present the intellectual stimulus craved by his searching mind. With another doctor he tried a partnership involving not only the practice of medicine but also the management of a general store, but soon settled into private medical practice in Plattsburgh. Three years later he was admitted into membership in the Clinton County Medical Society in New York State. At about this time Beaumont was offered a clerkship in the office of the Surgeon-General, who had been a military comrade, but declined it. That the call of military life persisted, however, is indicated by the fact that he was commissioned Surgeon in the New York State Militia in 1819 and the next year was appointed post surgeon in the Army by President Harrison and ordered to report to Fort Mackinac on Mackinac Island, Michigan.

On May 6, 1820, Beaumont left Plattsburgh, visited his family in Connecticut and returned to Albany, where he boarded a boat on the Erie Canal. He rode as far as Auburn, the western terminus of the canal, then continued on to Buffalo by stagecoach. Here he took passage on the steamer "Walk-in-theWater," the first steamboat on the Great Lakes, and on the last day of May, 1820, arrived in Detroit, where he stayed two weeks before continuing his journey. He reached Mackinac Island on June 16, 1820, and took charge of the hospital in the fort there.

The Indians, who pitched their wigwams on the lake shore, and the Canadian trappers and voyageurs returned to the post with their season's catch of furs at this time of year and Beaumont found himself amid scenes of confusion, wild dances, and drunken fights, far different from the well-ordered routine to which he had been accustomed in Plattsburgh. However, he readily adapted himself to the frontier life and, sensing the needs of the civilians in the post, he soon secured permission to engage in private practice in addition to his responsibilities at the fort.

From the journal in which he describes his journey to Michigan, one obtains further impressions of his character; at each stop he visited friends living in the locality as well as places of historic interest, usually attending church — Protestant or Catholic — if there was opportunity. One of his diversions on the long lake trip was to study Benjamin Franklin's autobiography, from which many guides to conduct were copied into his own journal. He spent much time, too, in reading and digesting the plays of Shakespeare, and other classics. However, despite his manifold activities at Mackinac, he was lonely, and after a year he returned to Plattsburgh and married Mrs. Deborah Platt, immediately returning with her to Fort Mackinac. Here she established a home in the New England tradition, a hospitable center of social activity for the small group of officers at the post.

Alexis St. Martin is one of the most celebrated experimental subjects in the history of physiology. He was employed by the American Fur Company as a voyageur at the time he was brought to the attention of Beaumont, who described him as "a Canadian, of French descent, at the above mentioned time about eighteen years of age, of good constitution, robust and healthy." On the 6th of June, 1822, in a tavern at the foot of the heights on which the fort was located, St. Martin was wounded in the abdomen by the discharge of a musket; "the contents entered posteriorly, and in an oblique direction forward and inward, literally blowing off integuments and muscles the size of a man's hand, fracturing and carrying away the anterior half of the 6th rib, fracturing the 5th, lacerating the lower portion of the left lobe of the lungs, the diaphragm, and perforating the stomach." The post surgeon was summoned; he dressed the wound, but held out little hope for the recovery of the patient. However, St. Martin did recover and after one year the wound had healed with the exception of the fistula in the stomach, and after 6 months more a fold of gastric mucous membrane developed and, acting as a valve, rendered external dressings unnecessary.

St. Martin was an invalid, without means of support, and out of charity Beaumont took him into his home, fed him, clothed him, and dressed his wounds, making every effort to close the gastric fistula. As the patient became stronger, Beaumont sensed the possible significance of the opportunity which chance had thus offered him, for he wrote, "This case affords an excellent opportunity for experimenting upon the gastric fluids and the process of digestion . . . Various kinds of digestible substances might be introduced into the stomach, and then easily examined during the whole process of digestion. I may, therefore, be able hereafter to give some interesting experiments on these subjects." Beaumont noted the behavior of the stomach after meals, looked into the stomach through the opening, put food and water into the stomach and withdrew them with a tube and made some preliminary observations on gastric digestion. However, the lack of facilities for experimentation and the isolation from contact with others having similar interests convinced him that he should transfer to a more populous part of the country. He was ordered to Fort Niagara in June, 1825, where experiments were continued on St. Martin. In July, Beaumont secured a two-month furlough and took St. Martin to Plattsburgh; first, however, exhibiting him in Burlington.

Experiment 1 in the first series of experiments in the book is dated August 1, 1825, which is the period of observations made at Plattsburgh; it may be that earlier results were corroborated here but it also appears that the experiments begun at Mackinac were extended at this time. It was observed that the *in vitro* digestion of meat in gastric juice was as rapid as that taking place in the stomach. The fact that the phenomenon of digestion occurred on the surface of the food was emphasized in these experiments. In September, St. Martin returned to his home in Canada, much to his benefactor's chagrin, and the experiments were thus terminated.

Beaumont now returned to Fort Niagara but soon was transferred to Green Bay, Michigan Territory, where he cared for the citizens of the surrounding country as well as for the

troops. Here he witnessed the Winnebago Indian uprising. His knowledge of and faith in vaccination is shown by his request (September, 1827) for a fresh supply of smallpox "infection" with which to immunize the people of Green Bay. In 1828, Beaumont moved to Fort Crawford, Prairie du Chien, on the Upper Mississippi, and the next year St. Martin, now with a wife and two children, returned to him at this post. Although St. Martin had worked hard as a voyageur during the intervening years, he seemed in good health and Beaumont records that "with his stomach in the same, or very similar condition as when he left me in September, 1825, I continued to prosecute the gastric experiments, which were commenced before he left me." Thus began what is recorded as the "Second Series" of experiments.

Beaumont had no laboratory equipment save a thermometer, a sand bath and some glass vials. On December 9, 1829, he began a study of the temperature of the stomach, meticulously recording the details of the weather — the cloudiness, the direction of the wind, the precipitation, the outside temperature — and after 8 experiments suggested "that the variations of the atmosphere produce effects upon the temperature of the stomach; a dry atmosphere increasing and a humid one diminishing it." Also, on the basis of one or two experiments, "it would seem that the heat of the stomach was increased during the active period of digestion." However, "subsequent experiments have not shown this result." At this time, too, his observations led him to conclude "that the stomach contains *no gastric juice,* in a free state, when aliment is not present," though later records indicate that he did find fasting juice. On March 13, 1830, he observed that the contact of bread crumbs with the gastric mucosa stimulated the flow of gastric juice. It was about this time also that he gave his classic description of fasting gastric juice: "It was clear and almost transparent; tasted a little saltish and acid, when applied to the tongue, similar to thin mucilage of gum arabic, slightly acidulated with muriatic acid."

During the "Second Series" (December 9, 1829, to April 9, 1831) he again demonstrated the similarity between *in vitro* and *in vivo* gastric digestion. He pointed out the failure of vegetable tissue to disappear as did meat and clearly indicated the difference in behavior of the clots of milk produced with gastric juice, on the one hand, and with acetic acid on the other hand. The *in vitro* experiments were "conducted with as much precision and integrity of observation as possible, with the temperature of the digesting bath kept as near blood heat as was practicable to regulate and continue artificial warmth — the thermometer varying, during the time, from 90° to 100°." In other experiments it was observed that whereas cold gastric juice had little digestive capacity, subsequent digestion could be induced by raising the temperature. This circumstance did not impress Beaumont as unusual for he says "there are but few chemical combinations that do not require caloric to effect their operations . . ." Experiments to elucidate the influence of bile on gastric digestion were carried out toward the end of this period but the results were inconclusive. During this time St. Martin "performed all the duties of a common servant, chopping wood, carrying burthens, etc., with little or no suffering or inconvenience from his wound." In the spring of 1831, with Beaumont's consent, he returned to Canada with his family.

Beaumont felt at this time that further progress in his study of gastric digestion required the collaboration of others better trained in chemical investigation than was he. He applied for and was granted a furlough for European travel but the Black Hawk War intervened and his leave was cancelled. At the end of hostilities, Beaumont and his family journeyed to Plattsburgh where, late in the summer of 1832, he was joined by St. Martin. That he was determined not to be disappointed again by the unexpected departure of his temperamental experimental subject is indicated by the fact that he drew up legal articles of agreement between himself and St. Martin, binding the latter for a year to "serve, abide, and continue with the said William Beaumont, wherever he shall

go or travel, or reside in any part of the world" and "to the utmost of his power, skill and knowledge, exercise and employ himself in and do and perform such services and business matters and things whatsoever as the said William shall from time to time order, direct and appoint . . . Said Alexis will at all times during said term, when thereto directed or required by said William, submit to, assist and promote by all means in his power such physiological or medical experiments as the said William shall direct or cause to be made on or in the stomach of him, the said Alexis, either through or by the means of the aperture or opening thereto in the side of him . . ." Beaumont also reserved to himself the right to "the exhibiting and showing of said Stomach, and the powers and properties thereof." Soon after this remarkable document was notarized, Beaumont and St. Martin went to Washington where Beaumont spent 4 very happy months. He read extensively in the literature bearing on the physiology of digestion and enjoyed the contact with scientific men, with diplomats and with military friends. The published accounts of his experiments brought him recognition and with it pleasant social contacts. But particularly he was pleased to be able to continue under favorable circumstances his experiments with St. Martin who, in the meantime, had been made a sergeant of a detachment of orderlies at the War Department in Washington.

The third series of experiments was begun in Washington on December 4, 1832. This continued the old line of investigation of the temperature of the stomach at rest and during digestion. Beaumont recorded the observation that "oil is particularly hard of digestion" and that, whereas mastication aids digestion, simple maceration of beef steak alone in the absence of gastric juice does not lead to digestion. He added gastric juice to putrefying meat, warmed it and noted the occurrence of digestion; the meat "lost its foetid smell and acquired a sharp acid or rather acrid taste," an observation reminiscent of that made by Spallanzani many years earlier. On January 14, 1833, he suspended a piece of hog's rib in fresh

gastric juice and recorded over a period of a fortnight the progressive loss of weight and the ultimate complete dissolution of the bone. The failure of saliva either to digest meat or to prevent its putrefaction was described. He concluded that "the sense of hunger resides in the stomach, as when previous steps have been gone through with." On January 26, 1833, he described the muscular movement of the stomach: "On settling the stem (of the thermometer) down into the stomach, a strong contraction of the muscular fibers was indicated, when the bulb had descended near to the pyloric end, by a sudden and peculiar movement of the tube, communicated to the thumb and finger that guided it . . . The stomach appeared to contract at that point forcibly, and grasp the bulb . . . This grasping sensation would continue for half a minute or more, and then appear to relax again." Again on January 30 he observed that the thermometer bulb, after being pulled toward the pylorus, rose again of its own accord. When put into the splenic end of the stomach, it "took a sort of irregular motion, revolving the tube from right to left, so as to turn it completely around, in the space of ten or fifteen seconds." On February 14, 1833, he made a comparison of the action of gastric juice on boiled beef with that of a mixture of hydrochloric and acetic acids on the same food; observing digestion with the former fluid and failure with the latter, he concluded that "probably the gastric juice contains some principles inappreciable to the senses, or to chemical tests, besides the alkaline substances already discovered in it."

During the sojourn in Washington in the winter and spring of 1833, Beaumont expanded his scientific horizon by making contact with Professor Dunglison of the University of Virginia, who talked with Beaumont in Washington and made a chemical analysis of the gastric juice. In one of his letters he wrote that after distilling off the acid, "the quantity of chloride of silver thrown down on the addition of the Nitrate of Silver was surprising." Dunglison also suggested some lines of research to Beaumont and it is likely that the experiments made in Washington reflected this assistance. Later Beau-

mont discussed his experiments with Benjamin Silliman the elder at Yale, who suggested that a sample of gastric juice be sent to Berzelius in Sweden for chemical examination. Beaumont's high hopes for some tangible assistance from these two distinguished scientists were not realized; after much delay the sample of gastric juice reached Sweden but seems to have received little attention from Berzelius. And Silliman's chemical examination of the fluid added little to the information already in Beaumont's hands. Beaumont felt that the time was ripe to publish the results of his experiments. He returned to Plattsburgh where, with the aid of his cousin, Dr. Samuel Beaumont, he prepared the manuscript for most of the book, at the same time conducting further experiments with St. Martin. This "Fourth Series" was the last recorded in his book; it was begun on July 9, 1833, and continued until November 1, 1833. These experiments seem to have been carried out to determine the effect of disease conditions on the activity and condition of the stomach and also to complete the information on the relative time required for the digestion of various foods. The data were added to the manuscript of the previous work and the section concludes with the regret that he was not able "to obtain returns from Professor Berzelius, to whom I transmitted, about seven months ago, a bottle of gastric juice for chemical examination. I could not, however, consistently with the expectations and wishes of my friends, further delay the publication of these experiments."

St. Martin appears to have remained with Beaumont during the winter of 1834. After a tour of inspection of military hospitals in April and May, 1834, Beaumont set out on a short trip to various cities in New England where he exhibited St. Martin to medical societies. Soon thereafter, St. Martin went again to visit his family in Canada; despite his promise to Beaumont he never returned to his benefactor, although for the rest of his life the latter continued his efforts to secure the services of St. Martin.

Despite the advice of friends that he secure a recognized publisher, Beaumont published his book himself and had it

printed in Plattsburgh, the first copies coming from the press in November, 1833. It is entitled "Experiments and Observations on the Gastric Juice and the Physiology of Digestion" and the heading continues "by William Beaumont, M.D., Surgeon in the U. S. Army, Plattsburgh. Printed by F. P. Allen, 1833." In the preface, the author states that his experiments "are made in the true spirit of enquiry, suggested by the very extraordinary case which gave me the opportunity of making them. I had no particular hypothesis to support; and I have therefore honestly recorded the result of each experiment exactly as it occurred . . . I had opportunities for the examination of the interior of the stomach, and its secretions, which has never before been so fully offered to any one." The introduction narrates the story of St. Martin's accident, recovery and association with Beaumont from 1822 until 1834. In addition, there are brief sections describing the method of withdrawing gastric juice, the valve which closed the fistula and the appearance of the interior of the stomach.

The part called "Preliminary Observations" is divided into 7 chapters; the first bears the title "Of Aliment;" it is brief and presents in a rambling way certain then current views on foods. In it the author states that he adheres to the ancient concept of the universal aliment, despite the fact that in a letter to him dated January 12, 1833, Professor Dunglison wrote that "it would be signally interesting to me to learn the comparative digestibility, as tested by this individual, of the great chemical divisions of aliments — amylaceous, mucilaginous, saccharine, aciduous, oily and fatty, caseous, gelatinous, and fibrous . . ." The second chapter, dealing with hunger and thirst, contains pertinent speculations, among which is the impression "that the sensation of hunger is produced by a distention of the gastric vessels, or that apparatus, whether vascular or glandular, which secretes the gastric juice . . ." Chapter three discusses satisfaction and satiety and the title of chapter 4 is "Of Mastication, Insalivation and Deglutition." In chapter 5, called "Of Digestion by the Gastric Juice," the views of the ancients are given, together with the conclusions

of Spallanzani, Tiedemann and Gmelin, Prout and others, showing that Beaumont was cognizant of the research of his time in gastric physiological chemistry. Of his own observations he noted that "when bile is found with the gastric juice, the acid taste is diminished . . ." and that "after the expulsion of the last particles of chyme, the stomach becomes quiescent, and no more juice is secreted, until a fresh supply of food is presented for its action, or some other mechanical irritation is applied to its internal coat." Chapter 6 discusses "The Appearance of the Villous Coat, and the Motions of the Stomach;" a graphic description is given of the character, extent and path of movement of the bolus after food enters the stomach, and the statement is made that "portions of chyme are constantly passing into the duodenum." The last chapter treats "Of Chymification, and Uses of the Bile and Pancreatic Juice." The remainder of the book is given over to the "Experiments and Observations, etc." already referred to and the various series of experiments are described in great detail.

The book attracted favorable attention both in this country and abroad. Beaumont was unable to secure a publisher in Great Britain, although Sir Andrew Combe, an English physiologist, published an English edition in 1838, but a German edition appeared in 1834 and a second American edition was published in Burlington in 1847. The appearance of the book established Beaumont's reputation as an investigator and throughout the remainder of his life seems to have influenced favorably his contacts with his medical colleagues. As a record of unselfish devotion to science, this document, after a century and a quarter, at once stirs feelings in the reader of admiration and humility. Beaumont had little scientific training as we now know it and much of his work was done with meager facilities and in intellectual isolation. Yet, because of his personal integrity, industry and desire to serve, his observations are regarded with confidence.

Shortly after the publication of his book, Beaumont was transferred to St. Louis to be stationed at the Arsenal, a

situation which suited his tastes inasmuch as it apparently permitted considerable latitude with respect to private practice. A little later (1836) he was offered the chair in surgery in the newly established Medical Department of St. Louis University; he was highly gratified to receive this honor but, in view of his connection with the Army, he felt it necessary to decline it. He believed that the opportunity for physiologic research presented by his association with St. Martin was in the nature of a public trust and he had the firm conviction that the monetary costs incident to the several series of experiments should be borne by the National Congress. He spent considerable effort in attempting to secure suitable remuneration on this account but was unsuccessful and came to the conclusion that, at least in diplomatic quarters, his investigations were unappreciated. Throughout the period of experimentation the Surgeon General had been deeply interested in the studies and had greatly facilitated their progress through various official channels. With a change in administration of this office, Beaumont, sensing that former privileged relations would not be continued, resigned his commission in the Army in December, 1839. Thus, at the age of 56 and after more than 25 years in military service, he again became a private citizen, remaining in St. Louis for the rest of his life.

Despite the demands of a very large medical practice, Beaumont never ceased to hope that St. Martin would return to him and that further experiments could be carried out. He kept in touch with his former experimental subject by frequent letters and sent emissaries to make contact with that "old fistulous Alexis," but though St. Martin desperately needed the proferred financial assistance, these efforts to bring him to St. Louis were fruitless. Except for the foregoing disappointment, Beaumont's later years were satisfying ones; he enjoyed the respect of his professional colleagues and was in demand as a speaker at medical meetings. In the spring of 1849 a severe epidemic of cholera swept the city; despite the fact that he was then 64 years old, Beaumont worked day and night until the scourge was conquered, a circumstance indi-

cative of his physical vigor at this time. In the spring of 1853 he suffered a head injury in a fall; he apparently was convinced that he would not recover, for in his illness during the following month he meticulously arranged his personal affairs so that they were in order when the end came on April 25, 1853. Thus ended the career of one whose life is an example of unselfish service and whose name will always assume an important place in the annals of American science.

ARTHUR H. SMITH

THE COMPOSITION OF SWINE MILK

II. THIAMINE, RIBOFLAVIN, NIACIN AND PANTOTHENIC ACID CONTENT [1]

VIRGINIA E. DAVIS, A. A. HEIDEBRECHT, ROBERT MacVICAR, O. B. ROSS AND C. K. WHITEHAIR

Oklahoma Agricultural Experiment Station, Stillwater

FOUR FIGURES

(Received for publication November 27, 1950)

Recent studies of the composition of milk have provided information concerning the B-complex vitamin content of the colostrum and milk of dairy cattle (Lawrence, Herrington and Maynard, '46; Pearson and Darnell, '46), goats (Holmes et al., '45), sheep (Pearson and Darnell, '46) and humans (Coryell et al., '45; Roderuck, Williams and Macy, '45; and Roderuck, Coryell, Williams and Macy, '45). Only fragmentary data concerning the composition of swine milk were available prior to the extensive investigations of Braude et al. ('47).

In the course of investigations in progress at this station (Heidebrecht et al., '50) samples of colostrum and milk from sows and gilts were collected. Advantage was taken of this opportunity to determine the thiamine, riboflavin, niacin and pantothenic acid content of swine milk. A summary of the principal results of these analyses for two successive lactations (spring and fall) is presented in the present paper.

[1] Published with the approval of the Director, Oklahoma Agricultural Experiment Station.

EXPERIMENTAL

Materials

The methods of management and feeding of the experimental animals have been previously described (Heidebrecht et al., '51b). The methods of collecting the samples of colostrum and milk were those described in the previous paper of this series (Heidebrecht et al., '51a). A portion of each milking was frozen and maintained at —14°C. until analyzed.

Methods

Total thiamine was determined essentially as described by Hodson ('45). A 10-ml sample of milk was digested with takadiastase at pH 4.5 for three hours at 50°C., clarified with trichloroacetic acid and filtered. The thiamine was oxidized to thiochrome, extracted with isobutanol and the fluorescence measured with a Coleman photofluorometer using an internal standard. Riboflavin was determined microbiologically by the method of Snell and Strong ('39), with minor modifications in the composition of the basal medium. The samples were not subjected to acidic or enzymatic hydrolysis since, in our experience, no material increase in the riboflavin content is observed following such treatment. Nicotinic acid was determined on whole milk by the microbiological procedures of Snell and Wright ('41). Pantothenic acid determinations were made by the methods of Barton-Wright ('45). In accord with the findings of Lawrence, Herrington and Maynard ('46) with cow's milk, enzymatic hydrolysis of samples of swine milk did not result in consistently higher values, indicating that only a small portion of the pantothenic acid is unavailable to *Lactobacillus casei.*

RESULTS AND DISCUSSION

The average thiamine, riboflavin, niacin and pantothenic acid content of colostrum and 5-, 15- and 55-day milk is presented in figures 1 through 4, respectively. In the interpretation of these results, the limitations of microbiological

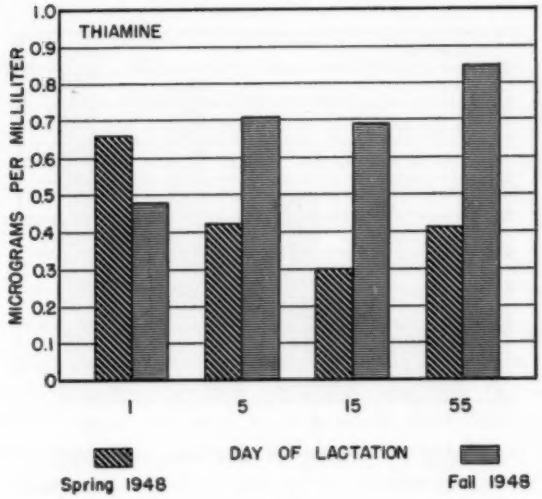

Figure 1

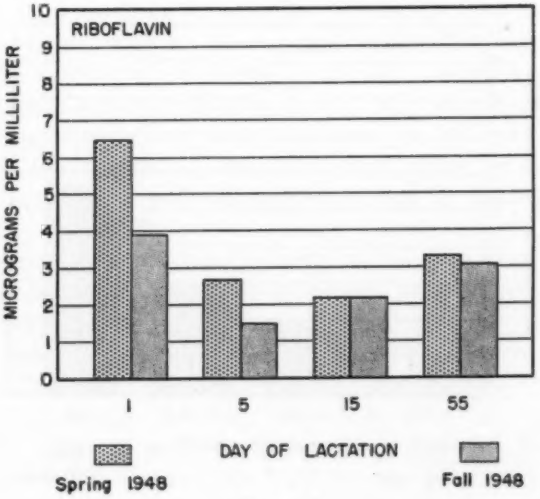

Figure 2

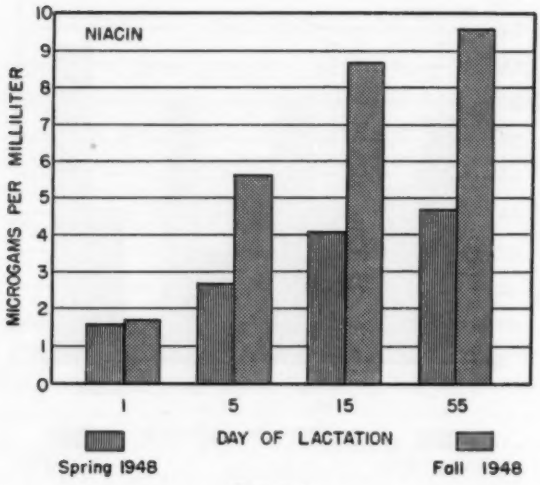

Figure 3

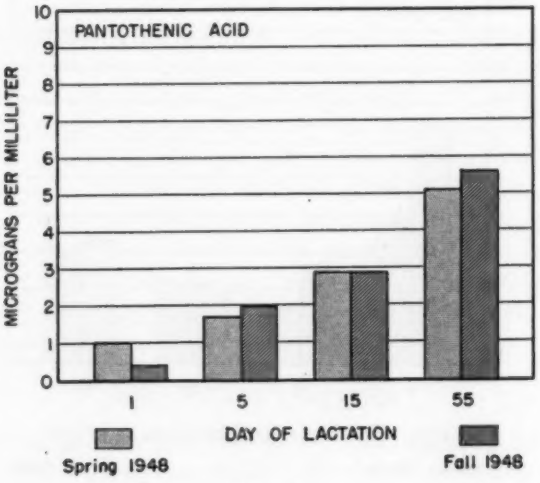

Figure 4

assay procedures should be kept in mind. In such determinations the error is usually assumed to be as high as 10%. In our experience with milk, the deviation between replicate analyses of the same sample assayed at different levels was usually less than this figure for riboflavin and niacin. The photofluorometric analysis for thiamine was also more accurate than 10%, but this will provide a conservative basis for comparison of the values presented.

Thiamine

The thiamine content of colostrum in the spring experiment was higher than that of the subsequent milk; this trend was similar to that observed by Braude et al. ('47) but the values obtained by these workers were considerably higher for both colostrum and milk. In the fall experiment, the reverse was true. No explanation is presently available for this anomolous observation; ration composition and management practices were essentially the same in both instances. Repeated checks were made in an attempt to rule out procedural analytical errors, with no change in results. Variations in the thiamine content of colostrum of a similar though less extreme nature were noted by Braude et al. ('47), who likewise were unable to account for such changes. In our observations, most of the values fall into the lower range, or below that of 0.52 to 1.01 μg per milliliter observed by Leuke, Duncan and Ely ('47). The ration fed by these workers, however, was definitely superior to the basal ration used in our studies, and the animals were allowed access to rye pasture prior to farrowing.

The thiamine in the milk showed no marked change after the 5th day, the following content, in micrograms per milliliter, being found: 5th day, $0.6 \neq 0.28$; 15th day, $0.6 \neq 0.28$; and 55th day, $0.7 \neq 0.33$. The values obtained in the fall experiment are comparable to those reported as total thiamine by Braude et al. ('47). The thiamine content of the fall milk was definitely superior to that observed in the pre-

vious experiment; again we are unable to present any reason for such a wide difference in composition. It is of interest, however, that these low thiamine and, as will be discussed later, low niacin values were associated with an outbreak of a severe gastroenteritis of unknown etiology which affected the suckling young. No explanation can be advanced for these observations; changes in the vitamin content of the ration were minimized by the fact that all ingredients came from a common lot in the spring and fall.

Riboflavin

The riboflavin content of swine colostrum was higher than that of the subsequent milk in both lactations, averaging $5.0 \neq 2.7$ μg per milliliter as compared with values of from 2 to 3 μg per milliliter for milk. In this respect the sow resembles the cow and the ewe (Pearson and Darnell, '46), whose colostrum contains appreciably more riboflavin than does the subsequent milk. The values observed in this study are similar to that (4.00 μg per milliliter) reported by Leucke and co-workers ('47) but much higher than those reported by the English group.

Subsequent to the first few days of lactation, the riboflavin content of milk appears to become stabilized and then rises somewhat throughout the remainder of the lactation period. Average values for all samples were, in micrograms per milliliter, as follows: 5th day, $2.1 \neq 0.9$; 15th day, $2.2 \neq 0.9$; 55th day, $3.1 \neq 0.7$. Again, these values are much greater than those reported by the group at Reading. In an attempt to resolve this obvious conflict in results, collaboration between the two groups of workers was undertaken. The results of these cooperative studies have been previously presented (Davis et al., '50) and require only brief comment here. It appears that riboflavin exists in swine milk in a somewhat different form than in cow's milk. Extraction procedures which removed riboflavin from cow's milk failed to effect more than a partial extraction from swine milk. Following incu-

bation of the milk with proteolytic enzymes (clarase and takadiastase), however, determination of the riboflavin in 10 representative samples of milk by a modification of the Conner and Straub ('41) procedure gave an average of 2.2 μg per milliliter, compared with a value of 2.6 by microbiological assay. Enzymatic treatment of a sample of cow's milk, however, did not result in any material increase in the apparent quantity of riboflavin, as evidenced by little change in the intensity of fluorescence.

Niacin

The niacin content of colostrum was 1.7 ≠ 0.5 μg per milliliter. This value was distinctly less than that of milk at any subsequent period during lactation. In this respect the sow resembles the ewe, the milk of which was found by Pearson and Darnell ('46) to contain about twice as much niacin as the colostrum. The only available data on the niacin content of swine milk is that of Leucke et al. ('47), who analyzed the colostrum of 6 sows and found a range of 1.14 to 1.85 μg per milliliter, with an average of 1.43.

The niacin content of milk was found to increase rapidly during the first few days. Values toward the end of lactation were only slightly higher than those for the 15-day milk. The following niacin content, in micrograms per milliliter, was found: 5th day, 4.3 ≠ 2.6; 15th day, 7.0 ≠ 3.9; 55th day, 8.0 ≠ 4.3. The content of this vitamin in the fall milk was nearly twice as high as that observed in the previous spring, although essentially the same basal ration and same management practices were being used. In the fall farrowing, however, the enteric disturbance in the pigs which prevailed in the spring was not observed. In order to obtain additional data on the possible relationship between the niacin content of the milk and the growth of the young, samples of milk were collected during the spring farrowing, 1949, on the 5th and 25th day post partum, from 15 sows and 13 gilts, and analyzed for this vitamin. The following results, in micrograms per

milliliter, were obtained: 5th day, sows, $4.4 \neq 1.9$; gilts, $2.7 \neq 1.9$; 25th day, sows, $6.9 \neq 2.9$; gilts, $6.7 \neq 2.9$. It will thus be seen that milk collected during the spring farrowing of 1949 resembled that of the preceding fall with respect to niacin content. During this spring season the herd was again relatively free of the enteric infection that had persisted throughout the farrowing period the preceding spring. Whether the lower niacin and thiamine content of the milk during this earlier lactation contributed in any way to the incidence of enteritis in the young pigs is unknown.

Pantothenic acid

Examination of the data presented in figure 4 shows that swine colostrum contained only a small amount of pantothenic acid, averaging $0.7 \neq 0.49$ µg per milliliter. This was slightly less than the 1.05 (0.60 to 1.70) found by Leucke et al. ('47). Swine colostrum was found to be lower in this nutrient than the values reported for either bovine or sheep colostrum.

There was a steady increase in the content of this vitamin throughout the whole period, so that near the termination of lactation swine milk contained more pantothenic acid than has been reported in cow's milk. The values observed were, in micrograms per milliliter: 5th day, $1.9 \neq 0.79$; 15th day, $2.9 \neq 1.80$; and 55th day, $5.4 \neq 3.04$.

Effect of ration

Since these studies were conducted incident to the more general problem of unknown nutritive requirements for reproduction and lactation in swine, the basal rations employed were presumably adequate with respect to known vitamins but deficient in some unknown factor or factors. Supplements were added primarily as a source of this factor, although they doubtless supplied limited amounts of the members of the B complex for which analyses were made. Careful scrutiny of the data failed to reveal any correlation between the various

supplements fed and milk composition. This should not be construed as suggesting that the vitamin content of swine milk is unaffected by ration, but rather that the particular supplements used in these experiments had so little effect that it was obscured by the rather great variability within treatments.

Effect of breed

Sows and gilts of Duroc, Chester White, Hampshire and crossbred breeding were used in these studies. Examination of the data failed to reveal any obvious correlations between breed and the vitamin composition of colostrum and milk with respect to any of the 4 B complex vitamins studied. Only a limited number of animals of each breed were available for study, however, and more extensive investigation will be necessary to eliminate the possibility of minor breed differences.

Effect of age

Both gilts and sows (from one to two years of age) were used in this study. Careful scrutiny of the raw data failed to reveal any consistent differences in the colostrum or milk of gilts or sows. Again, however, the number of animals was limited and small differences may actually exist.

SUMMARY

Colostrum and milk were obtained during two successive farrowing seasons from a total of 35 sows and gilts, and analyzed for thiamine, riboflavin, niacin and pantothenic acid. The average values from all samples of colostrum, in micrograms per milliliter, were: thiamine, 0.6; riboflavin, 5.0; niacin, 1.7; and pantothenic acid, 0.7. The average values for milk at the 5th, 15th and 55th days post partum, respectively, in micrograms per milliliter, were: thiamine, 0.6, 0.6 and 0.7; riboflavin, 2.1, 2.2 and 3.2; niacin, 4.3, 7.0 and 8.0; pantothenic acid, 1.9, 2.9 and 5.4. It was not possible to correlate the composition of colostrum or milk with respect to these vita-

mins and such factors as age, breed or supplementation of an all-vegetable ration with various materials presumably containing still unknown factors.

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A STUDY OF THE DISCREPANCY IN THE THIAMINE CONTENT OF MEAT AS MEASURED BY THE THIOCHROME AND RAT GROWTH METHODS

O. B. FARDIG,[1] N. B. GUERRANT AND R. A. DUTCHER
Department of Agricultural and Biological Chemistry, The Pennsylvania State College, State College

FOUR FIGURES

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The thiochrome procedure has been widely used in recent years in measuring the thiamine content of foods. When applied to many foods, thiamine values obtained by this method of assay are in good agreement with the results of bioassays. However, in the assay of meat and meat products thiamine values obtained by the thiochrome method are often considerably lower than those calculated from rat growth or rat curative data (Harris and Wang, '41; Lane, Johnson and Williams, '42; Guerrant, Vavich and Fardig, '45). Numerous explanations have been offered for this discrepancy. Some investigators (Lane, Johnson and Williams, '42; Guerrant, Vavich and Fardig, '45) have attributed the low thiochrome values to the incomplete extraction of the vitamin from the animal tissues; others (Harris and Wang, '41) have suggested the incomplete liberation of thiamine from cocarboxylase; and still others (Eppright and Williams, '44) have attributed the differences in the thiamine values obtained by the two methods of assay to incomplete adsorption

[1] Present address: Chemical Research Department, Bristol Laboratories, Inc., Syracuse, New York.

or elution of the vitamin in the chemical method. It has also been suggested that the higher thiamine content indicated by the biological method of assay is due to the stimulatory effect of thiamine degradation products (not measured by the thiochrome method), to the presence of unrecognized growth-stimulating materials in meat and meat products, or to the synthesis of biologically active materials in the intestinal tract of the rat when meat or meat products are fed (Brown, Hamm and Harrison, '43; McIntire et al., '43; Emerson and Obermeyer, '44).

Since the discrepancy in thiamine values obtained by the two methods of assay is more pronounced when these methods are applied to high protein products, especially to meat and meat products, and since meats are generally considered among the richer natural sources of thiamine, this problem seemed to us to merit further investigation.

EXPERIMENTAL

Methods

The thiochrome procedure used in the present investigation as a standard method of assay was that modification of the Hennessy procedure ('41) which had been employed by collaborating groups studying the nutritive value of canned foods (National Canners Association, '44). The procedure was as follows: The sample of ground meat was digested in 0.1 N sulfuric acid for one hour at 70°C., then buffered with sodium acetate and further digested overnight with takadiastase at 37°C. A portion of the filtered extract was adsorbed on and eluted from Decalso and the thiamine content of an aliquot of this eluate was oxidized to thiochrome. The intensity of the resultant fluorescence was measured by means of a model 12 Coleman electronic photofluorometer. The instrument was first calibrated by adding known quantities of thiamine to measured portions of the eluate, oxidizing to thiochrome, and measuring the increase in fluorescence due to the added thiamine. By this procedure, thiamine

values which varied less than 5% were obtained for replicate samples of meat.

The thiamine activity of the meat and meat products was also determined biologically by the rat growth method. The assay procedure used in this connection followed the general plan of Guerrant and Dutcher ('34), which involved the use of a purified diet supplemented with pure vitamins. The composition of the basal diet and the supplements used are given in table 1.

TABLE 1

Composition of basal diet

CONSTITUENTS	AMOUNT	VITAMIN SUPPLEMENTS	AMOUNT
	gm/kg		*mg/kg of diet*
Purified casein	180	Riboflavin	4.0
Salt mixture 185	40	Pyridoxine	4.0
Cellu flour	20	Calcium pantothenate	2.5
Sucrose (commercial)	710	*p*-Aminobenzoic acid	4.0
Cod liver oil (medicinal grade)	20	Niacin	50.0
Filtered butterfat	30	Inositol	10.0
		Biotin	0.1
		Choline	500.0

Weanling rats, housed in individual, all-metal, screened-bottom cages, were offered the basal diet during a 21-day depletion period and then arranged according to the best possible distribution of sex, litters, and weight into assay groups of 6 or more test animals. Following depletion, each animal was fed, as a supplement to the basal diet, a definite amount of thiamine, or of the material under assay, during a 21-day or 28-day test period. Records of food consumption and animal weights were made biweekly.

Materials

The meat used in this investigation was taken from a supply of 12-ounce cans of chopped ham [2] which had been prepared from a single batch of meat stock and which had been found to be relatively uniform in composition. However,

[2] Leeds.

to insure the uniformity of the product further, the contents of several cans were thoroughly mixed and then ground in a food chopper. The ground meat was resealed and stored in a deep-freeze unit until used. Subsequent studies showed the chopped ham to contain 5.2 μg thiamine/gm when assayed by the thiochrome method and 6.3 μg/gm when assayed by the rat growth method.

A quantity of the chopped ham was treated with sulfite following an adaptation of the procedure of Schultz, Atkin and Frey ('42). The excess sulfite was neutralized with a 10% hydrogen peroxide solution, using starch-KI-H_2SO_4 as an external end point indicator. After treatment, the meat was reduced to its original weight by drying at 60 to 70°C. in a forced-air oven. The sulfite-treated ham was stored in sealed containers at —18°C. until used. This material was found to contain no thiamine as determined by the thiochrome method of assay. An aqueous solution of thiamine hydrochloride, having a concentration of 25 μg/ml, was similarly treated with sulfite. No thiamine was detected in this solution when assayed by the thiochrome method.

Batches of the canned ham were also extracted and digested according to the thiochrome procedure and the resultant filtrates were concentrated at low temperatures for use in biological tests. The final concentration of these "ham extracts" was such that 1.5 ml of the extract were equivalent to the digested portion of 0.4 gm of the original ham. The thiamine content of these extracts was 1.4 μg/ml when measured by thiochrome assay.

The undigested residue remaining after preparing the extracts was collected, dried and also used for feeding tests. Two-tenths of a gram of this "ham extract residue" was calculated to be equivalent to the undigested portion of 0.4 gm chopped ham. According to the thiochrome assay, this residue contained no thiamine.

A "thiamine-free ham extract" was prepared by concentrating ham extracts which had been previously passed through Decalso to remove the vitamin. One and five-tenths

milliliters of this concentrate were equivalent to 0.4 gm ham. This extract was found to be devoid of thiamine when assayed by the thiochrome method. The Decalso employed in removing the thiamine from the meat extracts was washed with water and dried. After determining its thiamine content by thiochrome assay, appropriate quantities were fed to test animals in order to determine its thiamine activity.

Studies on the thiochrome method

The possibility of loss of thiamine in the thiochrome method of assay, owing to destruction of the vitamin or to its incomplete recovery, was investigated by assaying known quantities of the vitamin in the presence or absence of chopped ham. The recovery of thiamine from a solution of the pure vitamin averaged 99%, whereas the recovery of the vitamin when added to ham averaged 96%. Elimination of the adsorption and elution steps from the procedure resulted in no significant difference in thiamine values.

Modifications of the standard assay procedure were undertaken on the assumption that more drastic treatment of the ham might increase the liberation of thiamine. These modifications included variations in acid strength and in time and temperature of extraction. Also studied were the effects of preliminary digestions with pepsin, pancreatin, and castor bean lipase, as well as digestions with varying quantities of phosphate-affecting enzymes from a number of different sources.

Studies on rat growth

The completeness of the extraction procedure employed in the thiochrome assay for removing the vitamin from the animal tissue was tested by feeding depleted rats supplements of the concentrated "ham extract" and of "ham extract residue." Three groups of rats were employed in these tests. The rats of one group received 2.2 μg thiamine/day; those of the second group received 1.5 ml "ham

extract''/day; and those of the third group received 0.2 gm ''ham extract residue'' plus 2.2 μg thiamine/day. The average growth of the test animals comprising each group is presented in figure 1.

The biological activities of Decalso-treated ham extracts, and of the adsorbate, were determined. One group of thiamine-depleted rats received 2.2 μg thiamine/day, a second group received 1.5 ml ''thiamine-free ham extract'' plus 2.2 μg

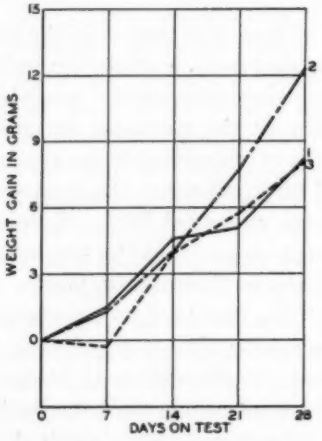

Fig. 1 Growth responses of rats receiving the following daily supplements: 1 — 2.2 μg thamine; 2 — 1.5 ml ''ham extract;'' 3 — 2.2 μg thiamine plus 0.2 gm ''ham extract residue.''

thiamine/day, those of a third group received 2.2 μg thiamine plus Decalso (thiamine-free), and a 4th group of depleted rats received daily supplements of the Decalso adsorbate in such quantities as to contain 2.2 μg thiamine by thiochrome assay. The results of these tests are given in figure 2.

The effects of supplements of sulfite-treated ham and of sulfite-treated thiamine were also studied. In this experiment, the rats composing one group were each given a supplement of 2.0 μg thiamine/day and the rats of a second group were fed a supplement of 2.0 μg thiamine plus 0.4 gm

sulfite-treated ham/day. The animals of a third group were given 2.0 μg thiamine plus 2.5 μg sulfite-treated thiamine/day. The responses of these animals are presented graphically in figure 3.

The influence of a sulfa drug on the growth responses of thiamine-deficient rats when thiamine, ham, or sulfite-treated ham was fed, was investigated by the addition of an insoluble sulfa drug to the basal ration, which was slightly modified

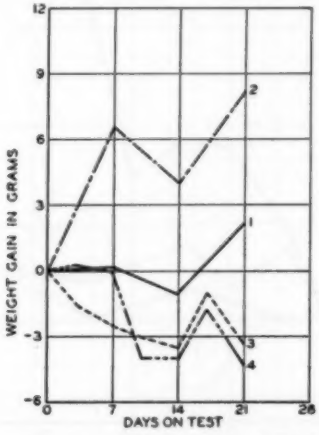

Fig. 2 Growth responses of rats receiving the following daily supplements: 1 — 2.2 μg thiamine; 2 — 2.2 μg thiamine plus 1.5 ml "thiamine-free extract;" 3 — 2.2 μg thiamine plus 0.6 gm Decalso; 4 — Decalso containing thiamine from ham (equivalent to 2.2 μg thiamine by thiochrome assay).

to include folic acid for these experiments. In this study, the growth of 6 groups of rats was compared. After depletion, three groups of rats were continued on the basal diet and the animals of each group were fed, respectively, daily supplements of 2.2 μg thiamine; 0.4 gm sulfite-treated ham plus 2.2 μg thiamine; or 0.4 gm chopped ham. The three remaining groups were fed the basal ration to which had been added 0.5% of phthalylsulfathiazole and the animals were given, respectively, the same supplements as the previous three

groups. The average growth responses made by the animals comprising the 6 groups are presented in figure 4.

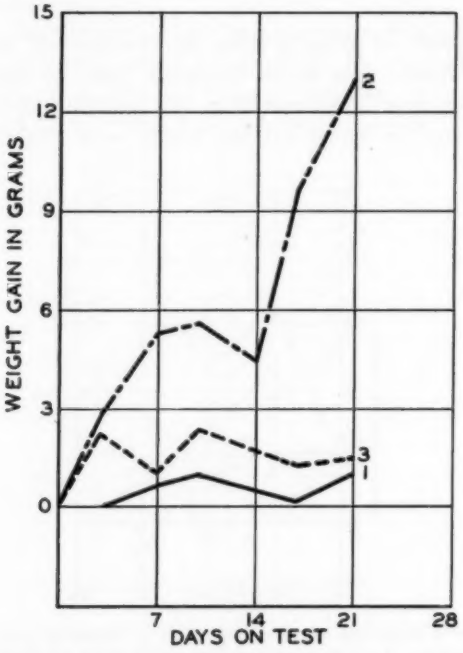

Fig. 3 Growth responses of rats receiving the following daily supplements: 1 — 2.0 μg thiamine; 2 — 2.0 μg thiamine plus 0.4 gm sulfite-treated ham; 3 — 2.0 μg thiamine plus 2.5 μg sulfite-treated thiamine.

DISCUSSION

The thiamine content of canned chopped ham as determined by the thiochrome method is significantly lower than that obtained by biological assay. This difference cannot be due to the destruction or incomplete recovery of free thiamine, since thiamine alone, and thiamine added to the meat product, was accounted for to the extent of 96 to 99% of its theoretical value when the standard thiochrome procedure of assay was followed. Efforts to increase thiamine values by modification

of the chemical extraction steps (on the assumption that part of the vitamin was in a combined form from which it is not readily liberated) were unsuccessful. Preliminary digestions with proteolytic and lipolytic enzymes, and modification of the phosphatase digestion, were also ineffective in increasing the thiamine values. These observations are in general agreement with those of Lane, Johnson and Williams ('42), and Hinman, Halliday and Brookes ('44). Houston et al. ('40),

FIG. IV

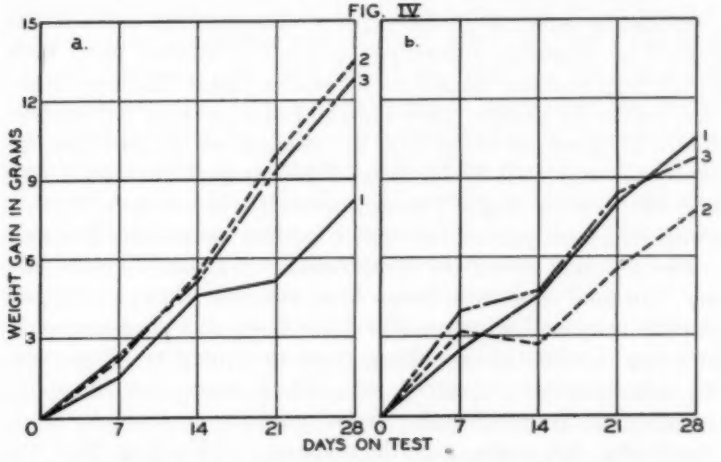

Fig. 4 Comparison of growth responses of rats (a) on basal diet only, and (b) on basal diet with 0.5% added phthalylsulfathiazole, receiving the following daily supplements: 1 — 2.2 μg thiamine; 2 — 2.2 μg thiamine plus 0.4 gm sulfite-treated ham; 3 — 0.4 gm ham (containing 2.1 μg thiamine by thiochrome test).

however, found slightly higher thiamine values in milk following a preliminary digestion with pepsin, and Jackson, Crook, Malone and Drake ('45) likewise observed higher thiamine values when pork was subjected to a preliminary digestion with pepsin.

Growth experiments with thiamine-depleted rats fed supplements of ham extracts prepared according to the procedure employed in the thiochrome assay reveal that all the growth-stimulating activity of the ham is contained in the

filtrate fraction and that none is found in the extracted ham residue (fig. 1). Daily supplements of the extract, which contained 2.1 μg thiamine by thiochrome assay, produced a greater growth response than did daily supplements of 2.2 μg of the pure vitamin. A statistical evaluation of the growth differences between control and experimental animal groups by the "t" test shows that the differences are significant.

The removal of thiamine from ham extract by its adsorption on Decalso resulted in only a partial removal of the growth-stimulating capacity of the extract, although the extract was devoid of thiamine according to the thiochrome test. Rats which received the thiamine-free extract plus thiamine grew at a markedly greater rate than did similar rats fed supplements of thiamine alone (fig. 2). Somewhat similar findings have been reported by Hinman, Halliday and Brookes ('44), who observed a slight thiamine activity in extracts of beef which had been passed through a column containing Decalso.

The animals given the supplement of Decalso containing the thiamine removed from ham extract gave a reduced growth response which might have been due to changes in intestinal absorption resulting from the quantity of the zeolite administered. Control rats, which were fed the same quantity of pure vitamin under similar conditions, made essentially the same growth response, indicating that the activity of the material taken up by Decalso was no greater than could be accounted for on the basis of its thiamine content as determined by thiochrome assay. It appears, therefore, that ham and ham extract contain a growth-promoting factor which is not adsorbed by Decalso under conditions favorable for the quantitative removal of thiamine.

The sulfite treatment of ham did not completely destroy its growth-stimulating activity for thiamine-deficient rats, although sulfite treatment completely destroyed the activity of thiamine (fig. 3). Inasmuch as sulfite-treated thiamine stimulated no growth, it may be assumed that the growth response resulting from feeding sulfite-treated ham is not due to a resynthesis of the vitamin from its sulfite degradation

products. Thus it appears that the growth response of thiamine-depleted test animals to ham must be due, in part, to the presence of an entity which is stable to sulfite under conditions where thiamine is destroyed. McIntire et al. ('43) observed that sulfite-treated liver had a growth-stimulating effect for rats receiving a suboptimum intake of thiamine.

Rats fed daily supplements of either 2.2 μg thiamine, 2.2 μg thiamine plus 0.4 gm sulfite-treated ham, or 0.4 gm ham (calculated to contain 2.1 μg thiamine according to thiochrome assay) show interesting growth responses (fig. 4a). The test animals receiving the ham and those receiving the thiamine plus sulfite-treated ham grew at similar rates over the entire test period, while the rats which received only the thiamine supplement grew at a slower rate. The growth-stimulating capacity of the ham, therefore, is greater than that induced by the thiamine, but almost equal to the activity of thiamine when the vitamin is supplemented with sulfite-treated ham. The difference in the growth responses due to feding the thiamine supplement and the thiamine plus sulfite-treated ham supplement was highly significant (one in 200). Assuming the growth-stimulating capacity of ham to be due, in part, to the presence of some active substance other than thiamine, the activity due to thiamine may be best evaluated by comparing the growth of rats fed supplements of ham with the growth of rats fed the pure vitamin and an additional supplement of sulfite-treated ham to supply the non-thiamine growth-stimulating material present in the untreated ham. When this comparison is made (ham compared with thiamine plus sulfite-treated ham), the thiamine in 0.4 gm may be estimated to be slightly less than 2.2 μg, or close to 5.5 μg/gm, a value which compares closely with that obtained by the thiochrome assay (5.2 μg/gm).

The inclusion of 0.5% phthalylsulfathiazole in the basal diet resulted in a greater reduction of the growth-stimulating capacity of ham and of sulfite-treated ham than of thiamine.

SUMMARY

The rat growth method of assay, when applied to chopped ham, yielded a significantly higher thiamine value (average 6.3 μg/gm) than did the thiochrome method of assay (average 5.2 μg/gm). The lower thiamine value obtained by the thiochrome method of assay was not due to the incomplete extraction of the vitamin or to loss of the vitamin during the assay, inasmuch as modifications of the extraction technic to insure more complete extraction did not increase the thiamine values, and reasonably good recovery of added thiamine was always achieved.

Ham extracts, thiamine-free ham extracts, and sulfite-treated ham were found to have greater growth-stimulating activity than could be ascribed to their thiamine contents as determined by the thiochrome method. Undigested ham residue remaining after extraction was found to be devoid of thiamine, as indicated by thiochrome assay, and also was ineffective in promoting growth in thiamine-deficient rats.

It appears that the growth-stimulating activity of chopped ham when fed to thiamine-deficient rats is due to two different factors. The more important of these factors is free thiamine or thiamine in a form readily liberated by the usual extraction or analytical procedure. The other factor present in the ham which stimulates growth in the thiamine-deficient rat exhibits properties different from those of the free vitamin. This latter factor is heat stable, is not inactivated by sulfite, is not adsorbed on Decalso, and does not fluoresce under conditions in which thiamine is oxidized to the fluorescent thiochrome. It is not definitely known whether this second growth-stimulating factor is in the ham per se or whether it is elaborated by the intestinal flora of the test animal as the result of ingesting the meat supplement, although a relationship with intestinal microorganism activity is definitely suggested by the reduced apparent thiamine activity of ham when the basal diet contains phthalylsulfathiazole. While the presence in ham of a factor other than thiamine which stimulates growth in thiamine-deficient rats

is suggested, the possible synthesis of thiamine or some other growth-stimulating factor in the intestines of the thiamine-deficient rat must also be considered.

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COMPOSITION OF SWINE MILK

I. MAJOR CONSTITUENTS AND CAROTENE, VITAMIN A AND VITAMIN C [1]

A. A. HEIDEBRECHT, ROBERT MacVICAR, O. B. ROSS AND C. K. WHITEHAIR

Oklahoma Agricultural and Mechanical College, Stillwater

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Because milk is the principal source of nutrients to the young during the lactation period, its major constituents have been determined for many species of animals. Until recently it has not been possible to obtain satisfactory milk samples from sows for chemical analysis. The stimulatory effect of injections of the oxytocic principle of the pituitary, however, has provided a means whereby representative samples of the whole milk can be readily obtained. Using such a technique, Braude et al. ('47) were able to obtain samples adequate for analysis, and the results of their investigations constitute the first reliable information on the composition of the milk of this species following a complete milking. More recently, Bowland and others ('49a, b) have studied the effect of the plane of nutrition on the composition of swine colostrum and milk.

In the course of an investigation at this station on the relation of nutrition to the reproduction and lactation performances of swine, samples of milk were collected to determine its chemical composition and nutritive value. It was found by Heidebrecht et al. ('50a) that rats made excellent growth on milk from sows that failed to raise a satisfactory number of pigs to weaning.

[1] Published with the approval of the Director, Oklahoma Agricultural Experiment Station.

This paper reports the percentage of major constituents and the levels of vitamins A and C in milk from sows of several breeds fed a variety of rations.

EXPERIMENTAL

Materials

An experiment designed to study the reproduction and lactation performances of sows confined to concrete pens and fed various rations has been reported by Heidebrecht et al. ('50b). For the present work the milk was collected from the sows in experiments 1, 2, and 3 of this study.

Colostrum and milk samples were obtained for chemical analysis in the spring of 1948 (experiment 1) from 15 Hampshire gilts producing their first litters and one Duroc sow producing her third litter. In the fall of 1948 (experiment 2) milk samples were collected from 9 Durocs, 5 Chester Whites, and 5 Hampshires producing their first or second litters. In experiment 3, the effect of vitamin A and carotene supplementation of the ration upon the vitamin A content of the milk was studied. Milk samples were obtained on the 5th and 25th days of lactation from 10 Duroc and three crossbred gilts, and 4 Hampshire, three Chester White and 8 Duroc sows. A detailed account of the methods of management, rations fed, and reproduction and lactation performances of the sows in each experiment has been given by Heidebrecht et al. ('50b).

Colostrum was obtained during parturition by manual expression of the udder. Milk samples were obtained on the 5th, 15th, and 55th days of lactation by manual expression following intravenous injection of 1 to 2 ml of pitocin.[2] The sows were separated from the pigs for a period of approximately two hours prior to milking. The samples were obtained by completely milking the entire udder. Carotene, vitamin A, and ascorbic acid determinations were made as soon as possible after sampling. The remaining portion of

[2] Parke, Davis and Co.

the sample was quick-frozen in sealed containers and analyzed later for total solids, solids-not-fat, fat, protein, ash, thiamine, riboflavin, niacin, and pantothenic acid. Colostrum samples were not analyzed for major constituents. The B-vitamin content of the colostrum and milk will be reported in a subsequent paper.

The following analytical methods were employed in this study: total solids, solids-not-fat, and ash were determined by the A.O.A.C. methods; fat by the Mojonnier extraction procedure; protein by the Kjeldahl method, the value for nitrogen being multiplied by the factor 6.38; carotene and vitamin A by a slight modification of the method of Boyer et al. ('44); and ascorbic acid by titration of a protein-free filtrate with a 2,6 dichloro-phenol-indophenol solution.

RESULTS AND DISCUSSION

The average values obtained for total solids, fats, solids-not-fat, crude protein, ash, carotene, vitamin A, and ascorbic acid are given in table 1.

TABLE 1

Composition of swine milk[1]

COMPONENT	COLOSTRUM		5TH DAY		15TH DAY		55TH DAY	
	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.	Mean	St. Dev.
Solids (%)			20	2.0	19	1.9	20	2.2
Solids-not-fat (%)			11	0.7	11	1.4	13	2.0
Fat (%)			9	1.7	8	1.5	7	0.9
Crude protein (%)			6	0.8	5	0.6	7	0.9
Ash (%)			0.9	0.09	0.9	0.09	1.3	0.05
Vitamin A (μg/100 ml)	132	70.7	33	19.1	22	6.2	19	6.6
Vitamin C (mg/100 ml)	19	7.0	13	4.0	11	2.0	11	2.6

[1] A minimum of 22 observations was used in computing the mean values and standard deviations in the above table.

Major constituents

In comparing the total solid content of the samples collected on the 5th, 15th, and 55th day post partum, the 15th day sample contained the lowest percentage of total solids, protein, and carbohydrate. The fat content decreased throughout lactation, while the ash increased from 0.9% on the 5th to 1.3% on the 55th day. The composition of the milk compared closely with that reported by Braude et al. ('47). The protein values were slightly lower than those reported by Bowland et al. ('49b).

The mean fat values of the milk were slightly higher than the values reported by Bowland et al. ('49a) and Hughes and Hart ('35). The values reported by the latter workers were based on the Minnesota Babcock method of analysis, whereas the Mojonnier fat extraction method was used in this study. Bowland et al. ('49a) reported that the Mojonnier method resulted in slightly higher fat values than the Babcock method of analysis.

Carotene

Carotene analyses were made only during experiment 1, 1948. The quantity of ether-soluble yellow pigment (as β-carotene) varied from 0 to 8.9 μg per 100 ml. In a majority of milk samples no measurable amount of pigment was present.

Vitamin A

The vitamin A content of the sows' colostrum was high; a marked decrease occurred, however, by the 5th day of lactation and a gradual decrease was noted from the 5th to the 55th days of lactation. The vitamin A value for colostrum from sows in the spring (experiment 1), 117 μg per 100 ml, was lower than during the fall (experiment 2), 145 μg per 100 ml. This is of interest because the sows in the fall (experiment 2) received a crude carotene concentrate at the level of 6,000 I.U. per pound of feed. However, for

the milk, the values found in the spring and fall were not consistently different.

The vitamin A content of colostrum and milk found by us was similar to that reported by Braude et al. ('46, '47) and by Bowland et al. ('49a, c).

Supplementation of the basal ration with carotene concentrates and vitamin A during experiment 3 afforded an

TABLE 2

The effect of carotene and vitamin A supplementation of the ration on the vitamin A content of sow's milk

LOT NO.	RATION	NO. OF SAMPLES	VITAMIN A			
			5th day		25th day	
			Mean	St. Dev.	Mean	St. Dev.
			μg/100 ml			
	Gilts — 1st lactation					
1	Basal	5	19	4.7	13	0.9
2	Basal + iodine	3	15	2.7	14	2.6
3	Basal + iodine + Vitamin A	5	43	8.0	30	7.1
	Sows — 2nd and 3rd lactations					
4	Basal + iodine + vitamin A	4	58	20.7	28	8.2
5	Basal + iodine + carotene	4	30	7.1	20	6.2
6	Basal + iodine + vitamin A + fish solubles	3	37	5.1	35	9.7
7	Basal + iodine + vitamin A + Merck's APF supplements 1 and 2	4	60	15.3	34	8.3
	Average for all gilts and sows					
	No vitamin A supplementation	8	18	4.6	13	1.7
	Vitamin A supplementation	16	50	15.7	31	7.7
	Carotene supplementation	4	30	7.1	20	6.2

opportunity to study the effect of such supplementation on the vitamin A content of colostrum and milk. The animals were fed, in addition to the carotene in the basal ration, 6,000 I.U of either vitamin A (high-potency fish liver oil) or carotene (crude carotene concentrate prepared from alfalfa) per pound of feed. The results of the analyses are presented in table 2. Supplementation with either carotene or vita-

min A was without effect on survival or weaning weights of young from these sows.

Milk from gilts and sows fed additional vitamin A was approximately three times higher in vitamin A on the 5th day, and twice as high on the 25th day, than milk from sows fed the unsupplemented rations. Milk from sows fed carotene was higher in vitamin A than milk from sows fed only the basal ration but lower than that of sows fed the preformed vitamin. Bowland et al. ('49c) reported that sows on pasture produced milk higher in vitamin A than sows in dry lot. Schofield et al. ('42) reported that the feeding of massive doses of vitamin A to sows late in pregnancy increased the vitamin A content of the colostrum.

Ascorbic acid

Swine colostrum was found to contain very large quantities of ascorbic acid, 19 mg/100 ml. The vitamin C content had declined by the 5th day of lactation to 13 and was stabilized at 11 in subsequent collections. These concentrations are greatly in excess of those found in the colostrum or milk of other domestic animals. They are, however, in close agreement with the values reported by Braude et al. ('47).

Effect of ration, breed, and age

It was not possible to demonstrate any correlation between the rations fed and the total solids, solids-not-fat, fat, protein, ash or ascorbic acid content of the milk. Considerable individual variation existed between animals within a given treatment, however, and this may have obscured such effects. Supplementation with either vitamin A or carotene increased the vitamin A content of both colostrum and milk.

No consistent differences in composition with respect to breed were found; but, again, considerable individual variation within a given breed was noted.

Gilts and sows of one and two years of age were used in these studies. Examination of the data failed to reveal

any correlation between the composition of the milk and age or number of previous lactations.

SUMMARY

Samples of swine colostrum and milk were collected and analyzed for total solid, solids-not-fat, protein, ash, carotene, vitamin A, and ascorbic acid content. The mean values for the principal constituents on the 5th, 15th, and 55th days of lactation, respectively, were, in percentage figures: total solids, 20, 19, 20; solids-not-fat, 11, 11, 13; fat, 9, 8, 7; protein, 6, 5, 7; ash, 0.9, 0.9, 1.3. The mean vitamin A content was, in micrograms per 100 ml: colostrum, 132; milk 5th day, 33; 15th day, 22; and 55th day, 19. Swine colostrum and milk contained essentially no carotene. The mean ascorbic acid content was, in milligrams per 100 ml: colostrum, 19; milk 5th day, 13; 15th day, 11; and 55th day, 11.

No correlation between the composition of milk and breed or age was found. Supplementation of the ration with materials presumably containing unknown factors was without effect. Addition of vitamin A or carotene, however, produced an increase in the vitamin A content of colostrum and milk.

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIVE VALUE OF MILK PROTEINS

I. EVAPORATED AND POWDERED MILKS

BESSIE B. COOK, AGNES FAY MORGAN, ELSIE O. WEAST AND JANICE PARKER

Department of Home Economics, University of California, Berkeley

ONE FIGURE

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The effect of heat treatment upon the biological value of the proteins contained in powdered and evaporated milk has been reported by several investigators. Fairbanks and Mitchell ('35) found a decrease of about 8% in the nutritive value of the proteins in choice, roller-processed powders and in preheated, spray-processed powders. If preheating was omitted in the spray-drying, this reduction did not occur. In the roller process the maximum temperature used was 134°C. for 4 seconds; the spray-dried milk was preheated at 60° and 63°C. and spray-dried at temperatures ranging from 93° to 149°C. Henry and Kon ('47) found no difference in the biological value of the proteins of dried whole milk whether preheated to 165°F. or maintained at 190°F. for 5 minutes before spray-drying. No comparison was made of these milks with raw milk.

Whitnah ('43) has stated that milk with high quality protein can be either (a) evaporated, canned, and sterilized or, (b) evaporated and irradiated to contain either 270 or 510 I.U. of vitamin D per quart and canned and sterilized, without loss of protein value as measured by biological value, digestibility, or growth of rats. The temperatures used for the sterilization process were not given. Hodson and Krueger ('46) noted a small loss of lysine in the evaporation but not in the

drying of milk. These workers attached no nutritional significance to the finding. For raw and evaporated milks, Henry et al. ('44) found biological values of 85.6 and 84.6, respectively, with but slight change in digestibility.

A reduction in the nutritive value of protein has been reported by Chick et al. ('35) for casein heated at 150°C. for 66 hours and for lactalbumin heated at 120°C. for 72 hours; the biological value of the lactalbumin was only slightly decreased, but the digestibility was lowered from 95 to 69%. Greaves, Morgan and Loveen ('38) reported a slight decrease in the digestibility of casein heated at 130° and 140°C. for 30 minutes, and a significant decrease in the growth value for rats. Weast, Groody and Morgan ('48) noted a decrease in the growth value for dogs of casein heated at 140°C. for 15 to 30 minutes, mainly because of decreased digestibility. Mader, Schroeder and Smith ('49) likewise found marked loss of nutritive value, for dogs, in autoclaved lactalbumin, which they also ascribed to decreased digestibility.

The clear-cut findings that heat, at the temperatures used in the experiments cited, causes a decrease in the nutritive value of the principal proteins of milk, casein and lactalbumin, and also of roller-dried and preheated, spray-dried milk powders, together with the more or less conflicting evidence concerning the effect of processing methods on the proteins of evaporated milk, have led us to further investigations in this field.

EXPERIMENTAL PROCEDURE

The protein efficiency (gain per gram of protein eaten) of fresh, evaporated, and dried milks was measured by the rat growth method of Osborne, Mendel and Ferry ('19). In the first group of experiments (table 2), the paired-feeding technique recommended by Mitchell and Beadles ('30) was used; in all other experiments, all of the rats in a group were fed equal amounts of protein, the amount being determined by the group eating the least. When necessary, a few grams of protein-free basal diet were fed to those animals that were not

satisfied with the amounts eaten by the rest of the group. This was seldom necessary, however, when whole milk was used as the source of protein. The rats were divided among the groups with the usual precautions as to distribution of litters, sex, and weight.

The composition of the diets is given in table 1. It was thought at first that 6% protein supplied by whole milk, containing lactalbumin which is of superior nutritional quality and rich in cystine, should be equivalent to 9% protein supplied by casein. A preliminary experiment, however, showed

TABLE 1

Composition of the diets

INGREDIENTS (PER CENT)	DIETS		
	A	B (basal)	D
Protein	6 [1]	..	9 [2]
Hydrogenated fat	20	20	20
Salt mixture [3]	2.5	4	4
Cornstarch	To make up to 100	76	To make up to 100
Cystine	0.1	..	..

[1] Supplied by evaporated or whole milk in an amount to give 6% protein. The milks were mixed with the other ingredients of the diet, spread in thin layers in shallow pans, dried by the use of electric fans, and pulverized.

[2] Supplied by powdered skim milk in an amount to give 9% protein.

[3] Hubbel, Mendel and Wakeman ('37).

that rats merely maintained their original weights on this diet and resumed normal growth only after the addition of cystine. Diet A, fed in the experiments reported in table 2, therefore, contained 0.1% of L-cystine. In the later experiments the level of protein was raised to 9% and cystine was omitted.

A mixture of cod liver oil and carotene was fed three times weekly to all of the rats in an amount which provided daily 133 and 18 I.U. of vitamins A and D, respectively. In the early experiments reported in table 2, the vitamin B complex was supplied daily as follows: thiamine hydrochloride, 10 μg; riboflavin and pyridoxine hydrochloride, each 20 mg; and an

TABLE 2

The efficiency of the proteins of whole fresh liquid milk, evaporated milk and the latter milk given additional heat treatment

EXPT.	KIND OF MILK [1]	NO. OF RATS	TOTAL WT. GAIN IN 28 DAYS	TOTAL PROTEIN INTAKE	PROTEIN EFFICIENCY [7] ± STANDARD DEVIATION	"t" [8] VALUE
			gm	*gm*		
I	(a) Whole fresh [2]	14	48	13.7	3.50 ± 0.11	
	(b) Evaporated [3]	14	47	13.8	3.40 ± 0.05	3.143 [9]
II	(a) Whole fresh [2]	15	51	14.7	3.47 ± 0.11	
	(b) Evaporated [4]	15	50	14.8	3.38 ± 0.05	2.924 [9]
III	(a) Evaporated [5]	8	46	16.3	2.82 ± 0.31	
	(b) Evaporated, heated [6]	8	43	16.3	2.63 ± 0.27	1.330

[1] These milks were incorporated into diet A as described in table 1.

[2] Locally purchased, fresh, pasteurized market milk.

[3] A locally purchased, commercial brand of milk.

[4] Prepared from one lot of milk by the usual commercial method of evaporation by Company A.

[5] Prepared from one lot of milk by the usual commercial method of evaporation by Company B.

[6] The same lot of milk described in footnote 5 but given additional heat treatment in a pressure cooker at 120°C. (15 lb. pressure) for 30 minutes.

[7] Protein efficiency is expressed as grams gain per gram of protein eaten.

[8] The "t" value according to Snedecor ('46), comparing the mean protein efficiency of the (a) and (b) milks in each of the experiments.

[9] A value of 2.779 is significant at the 1% level; larger values are highly significant.

equivalent of 0.15 ml of a concentrated extract of rice polish [1] as the source of the other members of the vitamin B complex. This mixture, when fed to weanling rats, gave growth comparable to that obtained by daily feeding of 0.5 gm of brewers' yeast, and contained half as much nitrogen. In the later experiments, when more of the crystalline B vitamins were available, it was possible to feed larger amounts of each of the vitamins without increasing the nitrogen intake from this source. The following mixture was fed: thiamine and pyrid-

[1] This was the concentrate marketed under the name "Galen B" by the Galen Company, Inc., Berkeley, California.

oxine hydrochloride, each 30 μg; riboflavin, 50 μg; calcium pantothenate, 100 μg; niacinamide, inositol, choline, and para-aminobenzoic acid, each 1 mg; and 0.1 ml of the rice polish extract. The adequacy of the first mixture was proved by the fact that no greater rate of growth resulted when the larger amounts of these vitamins were fed.

Comparison of fresh whole milk, evaporated milk, and the latter milk reheated

The nutritive quality of the proteins of whole, fresh, pasteurized milk was compared with that of the proteins of evaporated milk in experiments I and II (table 2). In order to avoid variations which might arise from the use of different milks, two local milk companies prepared, by their usual commercial procedures, enough evaporated milk for an entire experiment from one batch of milk. The evaporated milk used in experiment I was prepared by one company and that used in experiment II by the other. Since it was impossible to obtain whole, fresh, pasteurized milk from the same batches that provided the evaporated milks, this was purchased locally as needed. In both of these experiments the protein efficiency of the fresh, pasteurized milk was significantly higher than that of the evaporated milk.

The effect of heat treatment (120°C. for 30 minutes in a pressure cooker) on the nutritive quality of evaporated milk was measured in experiment III (table 2). The added heat caused a decrease in the protein value of the milk. The difference between the mean protein efficiencies of the unheated and heated samples was even greater than that between the whole fresh and evaporated milks, but statistically, because of the smaller number of rats in the experiment and the greater individual variations among the rats in each group, the former difference appeared less significant. That this difference was significant seemed obvious when these same rats were continued, after the initial 4-week period, on the same diets fed ad libitum for 15 additional months. While both groups of ani-

mals continued to gain weight steadily (fig. 1), those receiving the heated milk gained less rapidly, so that at the end of the 16-month period they were considerably smaller than the control group fed the unheated, evaporated milk. The condition of the rats, on the whole, remained good throughout the entire

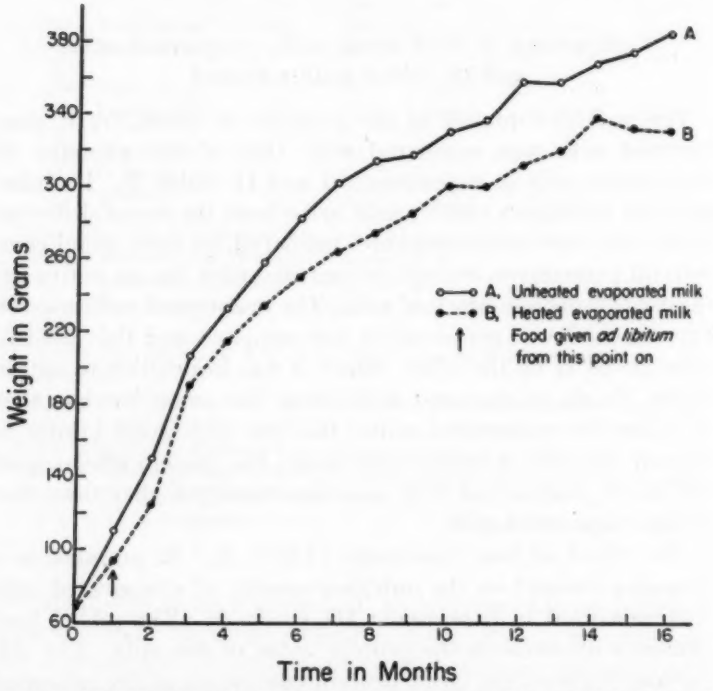

Fig. 1 Weight gains of rats fed for 16 months on diets containing 6% of protein supplied by unheated and heated (120°C. for 30 minutes) commercially prepared evaporated milk.

period, except that the animals which received the reheated milk had roughened and greasy fur.

Because there was some question of the validity of comparing whole, fresh milk of unknown origin with evaporated milk from another source, and also because the exact temperature

used in the sterilization of these milks could not be learned, a new set of experiments was carried out in which three evaporated milks were all prepared from one batch of fresh milk. One was unsterilized, the second was sterilized in the usual manner, and for the third the oxygen was exhausted from the can, replaced with nitrogen gas, and the milk then sterilized. The exact method of preparation of these milks and the results of the experiments are reported in table 3.

TABLE 3

The protein efficiency of unsterilized, sterilized, and sterilized gassed evaporated milks

EXPT.	DIET [1] AND TYPE OF EVAPORATED MILK [2]	TOTAL WEIGHT GAIN [3]	TOTAL PROTEIN INTAKE	PROTEIN EFFICIENCY ± STANDARD DEVIATION	"t" [4] VALUE
		gm	*gm*		
I	Freshly prepared:				
	1. Unsterilized control	47	13.9	3.38 ± 0.24	
	2. Sterilized	43	14.0	3.07 ± 0.30	2.636 [5]
	3. Sterilized, gassed	44	14.2	3.09 ± 0.18	3.144 [6]
II	Freshly prepared:				
	1. Unsterilized control	41	14.9	2.76 ± 0.26	
	2. Sterilized	39	14.8	2.63 ± 0.16	1.310
	3. Sterilized, gassed	37	14.7	2.51 ± 0.18	2.432
	Stored for 6 months:				
	1. Unsterilized control	38	14.7	2.58 ± 0.22	
	2. Sterilized	32	14.8	2.16 ± 0.32	3.444 [6]
	3. Sterilized, gassed	34	14.7	2.31 ± 0.21	2.792
	Stored for 1 year:				
	1. Unsterilized control	43	14.8	2.90 ± 0.27	
	2. Sterilized	40	14.8	2.70 ± 0.22	1.814
	3. Sterilized, gassed	39	14.7	2.65 ± 0.16	1.988

[1] Diet B, which was protein-free, was fed ad libitum.

[2] The evaporated milks were prepared by Dr. N. P. Tarassuk of the Division of Dairy Industry, University of California, Davis. They were measured by pipette into castor cups and fed 6 days of the week in an amount to provide daily about 0.5 gm of protein. In experiment I, all three milks were prepared from one lot of milk; in experiment II, except for the unsterilized control samples which had to be prepared from fresh milk for comparison with the others stored for 6 months and one year, one lot of milk was used. The entire amount of milk processed, evaporated, and canned by a standard commercial procedure was divided into three parts and handled as follows: 1 — Unsterilized control: frozen by immersion in ethyl alcohol and solid CO_2 at —29°C. and kept in freezing storage at —18°C. until fed; 2 — Sterilized: sterilized in the cans at 115–116°C. for 15 minutes; 3 — Sterilized gassed: was sterilized as in no. 2 above after the cans were opened, the air exhausted from the cans and replaced by nitrogen and the cans resealed.

[3] Average values for 10 rats in a group for 28 days.

[4] The "t" value according to Snedecor ('46), comparing the mean protein efficiency of the sample with that of the unsterilized control milk in the same group of experiments.

[5] Significant at the 5% level.

[6] Significant at the 1% level.

In experiment I the protein efficiencies obtained indicated that both sterilized milks, the ungassed and nitrogen gassed, had slightly but significantly less growth value than did the unsterilized control sample. In experiment II, another set of similarly prepared milks was made up in large enough amounts so that growth experiments could be made, not only when the milks were freshly prepared, but also after 6 months and after one year of storage at room temperatures. Again the results in all of the experiments indicated that the quality of the protein of the unsterilized control sample was significantly better than that of the sterilized samples, both ungassed and gassed with nitrogen, but in no case was there a significant difference between the sterilized gassed and ungassed milks.

Because of the necessity of preparing a fresh control sample of milk for comparison with milks stored for 6 months and for one year, and because the growth response of rats varies from one season to another, no conclusions were drawn concerning the effect of storage on the protein value. Since gas packing had been reported by Henry and Kon ('48) to exercise a slight protective effect on the protein efficiency of powdered skim milks after storage at a high temperature, it was thought likely that nitrogen, or the exclusion of oxygen, might afford a similar protection to evaporated milks. These workers also stated that the reaction responsible for the disappearance of the free amino nitrogen as a result of protein-lactose reaction does not require the presence of oxygen, but is accelerated by the presence of moisture. The findings in the present experiments with evaporated milk confirm those of Henry and Kon ('48) with powdered milks.

Skim milk powder

The results of experiments to determine the effect of varying the degree and time of preheating on the protein efficiency of skim milk powders are given in table 4. The methods used to prepare these milks are given in a footnote to this table.

Milk D was prepared by a method not used commercially; it was overheated to determine what may happen to the protein under such conditions. Milk A, which was not preheated, had a pronounced butyric acid odor at the end of the 28-day experimental period, even though it was kept at a low temperature. This was probably due to the presence of lipase, contained in

TABLE 4

The efficiency of the proteins in skim milk powders prepared with varying degrees of heat

TYPE OF MILK [1] AND DIET FED	NUMBER OF RATS	TOTAL WT. GAIN IN 28 DAYS	TOTAL PROTEIN INTAKE	PROTEIN EFFICIENCY ± STANDARD DEVIATION	"t" VALUE [2]
		gm	*gm*		
A	10	45	15.1	2.88 ± 0.27	
B	10	41	16.4	2.50 ± 0.25	3.386
C	9	39	15.9	2.45 ± 0.46	2.421
D	9	34	15.6	2.18 ± 0.33	4.880

[1] The powdered milks were prepared by Dr. H. Shipstead of the Division of Dairy Industry, University of California, Davis. They were prepared from one lot of milk as follows: Milk A, the unpasteurized control, was not preheated; milk B was preheated at 63°C. for 30 minutes; and milk C at 88°C. for 15 minutes. These three milks were then condensed to 38% and spray dried. Milk D was preheated at 93°C. for 15 minutes for stabilizing, precondensed to 17%, placed in 14-ounce cans where it was preheated at 93°C. for 18 minutes, and then placed in the regular sterilizer at 117°C. for 15 minutes. After cooling, the cans were opened and the milk was condensed to 38% and then dried on the spray drier. Each of these milks was incorporated in diet C as described in table 1.

[2] Mean values compared with those of the control group (fed milk A) as described in table 2. The "t" value for milk C is significant at the 5% level; for diets B and D a "t" value of 2.921 is significant at the 1% level. Values higher than these are highly significant.

the raw skim milk and not inactivated before drying. However, good growth was obtained with this milk. There were decreases in protein efficiency of 13 and 15%, respectively, in milks B and C, and of 24% in milk D. These changes are of the same order as those observed by Fairbanks and Mitchell ('35), and the loss in nutritive value seen in the proteins of milk B is comparable with that reported by these authors for spray-dried milk preheated at 60° to 63°C. Our milk D compared with their scorched sample, which showed a loss of 30%.

SUMMARY

The nutritive value of the proteins of two commercially prepared, evaporated milks has been shown, in two different rat growth experiments, to be slightly but significantly lower than that of whole, fresh, pasteurized milk.

It was found, by paired feeding for 4 weeks, that the growth of rats was less on evaporated milk given additional heat treatment at 120°C. for 30 minutes than on the same milk unheated. These rats, when continued on the same diets ad libitum, grew steadily, but the difference in weights between the two groups was somewhat increased at the end of 16 months.

The protein efficiencies of evaporated milk sterilized at 115 to 116°C. without removal of the oxygen, and of milk in which the oxygen was replaced with nitrogen and the milk subsequently sterilized, were not significantly different from one another, but both were significantly lower than the protein efficiency of the same evaporated milk unsterilized. Storage for one year at room temperature did not alter the value of the proteins of these sterilized milks. The changes which took place in the protein (probably a protein-lactose reaction) did not appear to be dependent upon the presence of oxygen.

The efficiency of the protein of preheated skim milk powders, when compared with that of a powder which was not preheated, was decreased in proportion to the degree and time of heating.

These studies justify the conclusion that the ordinary sterilization or preheating temperatures used in the preparation of evaporated and powdered skim milks decrease the protein efficiency of the milks in proportion to the degree of heat used. While the damage is not marked at the temperatures commonly used, it may be increased by careless overheating in commercial preparation of the products and in cooking.

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIVE VALUE OF MILK PROTEINS

II. RAT GROWTH STUDIES WITH CASEIN AND LACTALBUMIN AND THEIR LACTOSE DERIVATIVES

BESSIE B. COOK, AGNES FAY MORGAN, BEATRICE SINGER AND JANICE PARKER

Department of Home Economics, University of California, Berkeley

ONE FIGURE

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In report I of this series (Cook, Morgan, Weast and Parker, '51), data are given which show that preheating of powdered skim milk at 63°C. for 30 minutes or at 88°C. for 15 minutes, and sterilization of evaporated milks at 115 to 116°C. for 15 minutes, caused a slight decrease in the protein efficiency of the proteins, when measured by the rat growth method.

Greaves, Morgan and Loveen ('38) reported a slight decrease in the digestibility of casein heated at 130° and 140°C. for 30 minutes, and a significant decrease in the growth value for rats. When lysine was fed with the heated casein, the growth value was partially restored; histidine provided some additional improvement. Weast, Groody and Morgan ('48) found inferior growth, with decreased digestibility and nitrogen retention, in dogs fed casein heated at 140° for 30 minutes or 200°C. for 15 minutes. The addition of lysine to the diet partially compensated for the damage to the casein by heat.

Patton, Hill and Foreman ('48) found partial amino acid inactivation in casein refluxed in a 5% glucose solution for 24 hours at 96.5°C. McInroy et al. ('49) found, with dogs, a much greater decrease in digestibility and nitrogen retention

when casein was mixed with dextrose and autoclaved than when it was autoclaved without the dextrose. They attributed the damage to the protein to a reaction among certain essential amino acids and the sugar. Moisture intensified the reaction.

Several studies have been made on the effect of heat on the biological value of lactalbumin. Chick et al. ('35) reported that the biological value of lactalbumin heated at 120°C. for 72 hours was only slightly decreased, but that the digestibility was lowered from 95 to 69%. Neither the method of preparation of the lactalbumin nor the lactose content, if any, was given. Davis, Rizzo and Smith ('49) reported considerable decrease in the growth of rats fed lactalbumin [1] heated by dry heat at 140°C. for 60 minutes. When heated at 200°C., or when autoclaved at 120°C., for 60 minutes, there was extensive browning of the lactalbumin and its growth value was destroyed. Lysine did not repair the damage. In a later report on nitrogen balance in dogs, Mader, Schroeder and Smith ('49) found that the digestibility and biological value of this lactalbumin were less affected by heat after removal of the dialyzable constituents (lactose). They also believed that the decrease in the nutritive value of the protein was due to the interaction of lactose with the protein.

Three experiments were carried out to investigate further the changes which take place when milk is subjected to heat treatment: (a) feeding various essential amino acids in an effort to determine which ones in lactalbumin and casein are destroyed or rendered unavailable by heat or lactose or both; (b) determining the amount of lactose which may be combined with casein and lactalbumin under different conditions of reaction; and (c) measuring the effect of added lactose or heat or both on the value of these two proteins for rat growth.

EXPERIMENTAL METHODS

Male and female weanling rats selected, caged, and fed as described in paper I (Cook, Morgan, Weast and Parker, '51),

[1] Borden's Labco (15-42).

were used for the growth experiments. In the experiments in which the diets were supplemented with amino acids, the paired-feeding technique of Mitchell and Beadles ('30) was used. In all other experiments, instead of paired feeding, an attempt was made to feed all of the experimental groups the same amount of food. This can be successful only when the rats are all of the same age and there is but small variation in weight. At the beginning of the experiments, before the effect of heat on the protein became evident, the intakes were almost identical; but when the rats receiving the diets containing the heated protein began to eat less, the amount of food offered to the control groups had to be restricted accordingly. Ordinarily it was possible, in this way, to control the total protein intakes of all the animals for the entire feeding period to within $\pm$ 1 gm. However, in the few cases where the growth value of the protein was almost completely destroyed and there was almost complete refusal of food by an entire group of rats toward the end of the experiment, the other groups were not restricted to this level of intake.

The diets used were of the following percentage composition: salt mixture,[2] 4; hydrogenated vegetable fat, 20; protein, 9 when supplied by lactalbumin and 12 when supplied by casein; and cornstarch to make up to 100. Vitamin supplements fed separately provided daily: vitamin A, 133 I.U.; vitamin D, 18 I.U.; thiamine and pyridoxine hydrochloride, 30 µg each; riboflavin, 50 µg; calcium pantothenate, 100 µg; 1 mg each of nicotinamide, para-aminobenzoic acid, choline, and inositol; and 0.1 ml of a rice polish concentrate [3] as a further source of the entire vitamin B complex.

The lactalbumins used for the experiments are described in table 1. The casein used was an acid-precipitated, dried commercial product designated "raw casein."

The two soluble lactalbumins and casein were chemically combined with lactose under both mild and severe conditions

[2] Hubbel, Mendel and Wakeman ('37).

[3] The rice polish concentrate is marketed under the name "Galen B" by the Galen Company, Inc., Berkeley, California.

of treatment. The combination of the proteins with lactose, referred to as the mild lactose treatment, was carried out at 52°C. for 18 to 24 hours, using the proportions of 1 gm of protein to 300 mg of lactose, 6 ml of distilled water and 2 ml of 0.2 M phosphate buffer at pH 7.6. The solutions were dialyzed in running tap water and then in distilled water, concentrated in an air current, and lyophilized.

TABLE 1

Description of and symbols used to designate the different lactalbumins

SYMBOL	PROTEIN (N × 6.38)	ASH	REDUCING SUBSTANCE [4]	COLOR	SOLUBILITY IN WATER	pH OF WATER DISPERSION
A [1] (soluble)	77.5	10.30	0.00	White	Soluble	....
A (35–1)	81.9	1.75	2.33	Tan	Insoluble	4.2 in 10% dispersion
A (35–2)	91.0	1.64	0.00	Tan	Insoluble	6.7 in10% dispersion
B [2] (15–42)	76–83	2.0–4.0	2.50–4.25	Tan	Soluble	4.5–4.7 in2% suspension
Ethanol precipitated [3]	75–80	0.3	0.00	White		....

[1] A — provided by the Avoset company of San Francisco, California. The values given are those determined by the Avoset Company.

[2] B(15–42) — Borden's lactalbumin. The values given are from the booklet "Labco Products for Biological Research," the Borden Company, New York, 1941.

[3] Laboratory-prepared ethanol alcohol precipitated from whey supplied by the Dairy Industry Division at the University of California, Davis. The whey was concentrated to one-sixth of its original volume and saturated with magnesium sulfate. It was then filtered, the filtrate dialyzed in running tap water until it was salt and lactose-free, concentrated by suspending it in the dialysis tubing in the air current from an electric fan, and precipitated at a 60% ethanol concentration with cold 95% ethanol.

[4] Lactose.

The severe lactose treatment, in which the proteins were refluxed in a 5% lactose solution at 95°C. for 24 hours, was carried out under essentially the same conditions as those described by Patton, Hill and Foreman ('48).

The heated proteins were prepared by placing them in shallow pans, in a layer approximately one-fourth inch thick,

in an air oven at temperatures ranging from 100° to 150°C. They were heated for 30 or 70 minutes, and stirred frequently to prevent uneven browning.

RESULTS AND DISCUSSION

Lactalbumin

A series of experiments using the lactose-containing lactalbumin described in table 2 was designed to test the minimum amount of heat required to cause a significant decrease in the

TABLE 2

The protein efficiency (p.e.) of a lactose-containing lactalbumin [1] which was unheated, or heated for 30 minutes at varying degrees of temperature

CONDITION OF LACTALBUMIN	TOTAL WEIGHT GAIN [2]	PROTEIN INTAKE	P.E. AND STANDARD DEVIATION	DECREASE IN P.E. BY HEAT	"t" VALUE [3]
	gm	*gm*		%	
Unheated	48	20.0	2.40 ± 0.13	..	
Heated at 100°C.	49	20.0	2.45 ± 0.29	0	0.570
Heated at 120°C.	42	20.0	2.10 ± 0.25	12	3.970
Heated at 130°C.	39	20.1	1.94 ± 0.24	19	6.341

[1] Borden's Labeo (15–42).

[2] Average gain in weight of 14 rats in a group for 28 days.

[3] Comparison of the mean protein efficiency of the unheated lactalbumin with that of each of the heated lactalbumins by the method of Snedecor ('46). For 26 degrees of freedom, 2(n—1), a value of 2.770 is significant at the 1% level. Values of "t" greater than this are highly significant.

protein efficiency of this product. Diets containing 9% protein supplied by the unheated lactalbumin and by that heated in an air oven, at temperatures of 100°, 120° and 130°C. for 30 minutes, were fed to rats for 28 days. Heating at 100° caused an insignificant change in the protein efficiency, but there were efficiency decreases of 12 and 19% in the lactalbumins heated at 120° and 130°C., respectively. This is noteworthy when one considers that baking temperatures often exceed 120°C. and that sterilization temperatures used for evaporated milks are usually around 117° to 120°C. (243 to 248°F.).

Five rats from the group fed unheated lactalbumin and 5 from the group which received lactalbumin heated at 130°C. were kept on the respective diets for 12 months (fig. 1). After the first 42 days they were fed ad libitum, but records of the

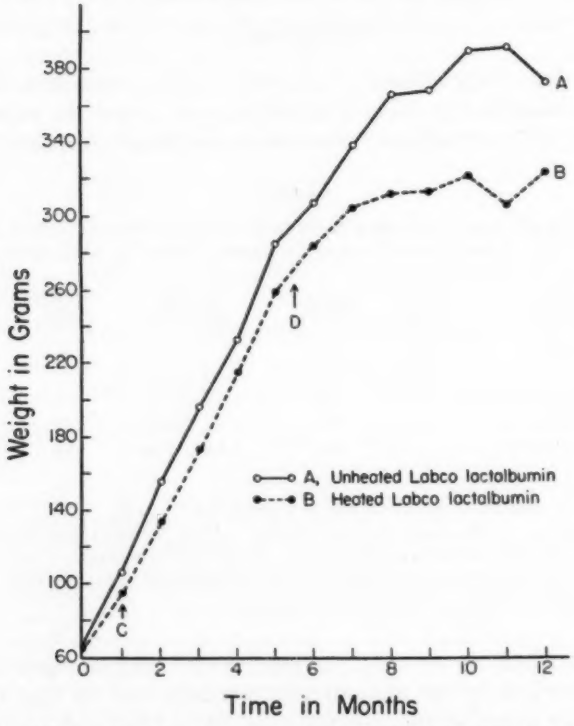

Fig. 1 The growth of 5 rats per group on diets containing 9% protein supplied by B(15-42) lactalbumin, unheated or heated. C: beginning of ad libitum feeding. D: lactalbumin heated at 140°C. for 30 minutes replaced that heated at 130°C.

food intakes were kept. At the end of 22 weeks the lactalbumin was heated at 140°C. instead of 130°C. in order to exaggerate any effect which might be obtained by the long-continued feeding. In spite of the facts that the food intakes

were no longer restricted and that the rats could eat a sufficient quantity of the heated lactalbumin diet to overcome the difference in quality between the heated and unheated protein, they failed to do so. Both groups continued to gain weight slowly for about 9 months, after which the weights declined or were extremely variable and the difference between the groups became even greater. The conditions of coat and eyes of the group given the heated diet were clearly less favorable than those in the other group, and there was increased susceptibility to respiratory infections. These findings are in accordance with those of a similar experiment in which evaporated milks, unheated and heated at 120°C. for 30 minutes, were fed for 16 months (Cook, Morgan, Weast and Parker, '51).

In an attempt to determine which amino acids [4] may have been inactivated in the lactalbumin as a result of heat treatment, diets containing 9% unheated or heated lactalbumin [5] were supplemented with the following: L-lysine dihydrochloride and L-histidine dihydrochloride, singly and together; DL-isoleucine; L-valine; DL-leucine; DL-threonine; DL-methionine; DL-norleucine; L-arginine; and L-ornithine. These diets were fed to litter-mate pairs of rats. One rat of the pair was fed the unheated protein and the other the heated; each group, except the control groups for each experiment, received the amino acid supplement. The results of these experiments may be found in table 3.

Though several of the experiments contained too few pairs of rats to lend great significance to the results, all of the groups showed the usual effect of heat on the nutritive value of the protein. In experiment I, when the lactalbumin was heated at 130°C. for 30 minutes, the decrease in protein efficiency appeared to be of doubtful significance. However, in the previous experiments reported in table 2, when there

[4] Merck and Company, Inc., Rahway, New Jersey, generously contributed most of the amino acids in this study.

[5] See footnote 1, page 64.

TABLE 3

The effect on growth of rats, as determined by the paired-feeding method, of the supplementation of diets containing 9% of unheated or heated lactalbumin [1] with various amino acids

EXPERIMENT AND GROUP NUMBER		NUMBER PAIRS IN GROUP	AMINO ACID SUPPLEMENT [2]	UNHEATED LACTALBUMIN			HEATED [5] LACTALBUMIN			
				Total weight gain [3]	Protein intake	P.e.[4] with standard deviation	Total weight gain [3]	Protein intake	P.e.[4] with standard deviation	"t" [6] value
				gm	*gm*		*gm*	*gm*		
I	1	5	None	67	30.9	2.16 ± 0.23	59	30.8	1.93 ± 0.16	1.924
	2	4	L-histidine	69	33.6	2.05 ± 0.28	60	33.4	1.80 ± 0.23	1.380
	3	5	L-lysine	71	31.5	2.25 ± 0.14	61	31.5	1.92 ± 0.25	1.538
	4	4	L-histidine and L-lysine	62	32.5	1.90 ± 0.11	60	32.1	1.87 ± 0.18	0.273
II	1	6	None	55	22.2	2.48 ± 0.31	48	22.4	2.14 ± 0.22	2.189
	2	6	L-lysine	59	21.4	2.77 ± 0.21	47	21.4	2.19 ± 0.28	4.696 [7]
	3	6	DL-isoleucine	57	22.5	2.53 ± 0.32	48	22.2	2.16 ± 0.49	1.547
	4	6	L-valine	65	23.4	2.77 ± 0.31	55	23.4	2.35 ± 0.27	2.482 [8]
	5	6	DL-leucine	64	22.7	2.80 ± 0.31	51	22.7	2.24 ± 0.17	3.864 [7]
III	1	9	None	56	23.5	2.38 ± 0.24	44	23.6	1.86 ± 0.16	4.789 [7]
	2	4	DL-threonine	66	24.1	2.74 ± 0.27	54	24.1	2.24 ± 0.14	3.377 [7]
	3	4	DL-methionine	63	23.5	2.68 ± 0.24	53	23.7	2.23 ± 0.25	2.597 [8]
IV	1	7	None	58	25.5	2.27 ± 0.25	48	25.5	1.84 ± 0.34	2.689 [8]
	2	6	DL-norleucine	58	25.5	2.27 ± 0.32	47	25.5	1.84 ± 0.33	2.305 [8]
	3	6	L-arginine	75	30.0	2.50 ± 0.18	62	30.0	2.07 ± 0.19	3.859 [7]
	4	6	L-ornithine	60	26.2	2.29 ± 0.14	55	26.2	2.10 ± 0.25	1.637

[1] Borden's Labco (15–42) lactalbumin was used.
[2] All of the amino acids were added at the level of 0.2% except DL-threonine, which is active only in the natural form and was fed at 0.4% of the diet.
[3] The feeding periods in experiments I, II, III, and IV were 42, 28, 35, and 35 days, respectively.
[4] P.e., protein efficiency.
[5] In experiment I the lactalbumin was heated at 130°C. for 30 minutes; in all others it was heated at 140°C. for 30 minutes.
[6] The "t" value according to Snedecor ('46), comparing the mean protein efficiency of the unheated lactalbumin with that of the heated lactalbumin.
[7] Significant at the 1% level.
[8] Significant at the 5% level.

were 14 rats in a group, the decrease due to heat treatment of the lactalbumin at this temperature was highly significant.

In order to exaggerate still further the damage to the nutritive value of the protein, the lactalbumin was heated at 140°C. for 30 minutes in experiments II, III, and IV (table 3). In experiment II the difference between the mean protein efficiencies of the unheated and heated proteins was of doubtful significance; in experiments III and IV, the differences were significant at the 1% and 5% levels, respectively.

Histidine appeared to depress the growth value of both the unheated and heated lactalbumins. With lysine added, histidine appeared to depress the unheated but not the heated protein, so that the value of the two proteins appeared about equal but not equivalent to that of the raw unsupplemented lactalbumin. The effect of lysine alone was tested in experiments I and II, and both times there was slight, but not significant, improvement in the unheated lactalbumin and no change in the heated product. These findings are different from those obtained with casein (Greaves, Morgan and Loveen, '38) in that addition of lysine significantly improved the growth value of the casein heated at 130° and 140°C., and histidine also slightly improved that heated at 140°C. The findings are in agreement, however, with the results of Davis, Rizzo and Smith ('49), who found that neither lysine nor histidine improved the protein value of heated lactalbumin.[6]

Neither norleucine nor isoleucine produced a significant change in the nutritive value of the proteins of either the unheated or the heated lactalbumins.

Valine, leucine, threonine, methionine, and arginine were each found to produce slight improvement in both the unheated and heated lactalbumins. In no case, however, was the amino acid-supplemented protein significantly better than the corresponding unsupplemented protein, but a significant difference was maintained between the heated and unheated proteins regardless of the amino acids added.

[6] See footnote 1, page 64.

[1] Significant at the 5% level.

Ornithine, on the other hand, produced improvement only in the heated lactalbumin, but the heated, supplemented protein was not significantly better than the unsupplemented, heated product. This amino acid was tried in previous experiments (unpublished data), where it supplemented heated casein about one-half as well as did lysine.

This difference in response to arginine and ornithine would appear to indicate the existence of some function of the latter aside from its role in relation to arginine production and urea synthesis. Perhaps the subnormal growth of young rats fed diets containing only mixtures of isolated amino acids without arginine (Rose, '38) may be due to failure of the ornithine supply. According to Borsook and Dubnoff ('41), arginine production from citrulline, by kidney slices, is dependent on the presence of glutamic acid or its precursors, one of which is ornithine. If the supply of these substances is reduced in the heated protein, arginine production, upon which the animals depend, may be decreased. It follows that any of the glutamic acid precursors — aspartic acid, glutamic acid, proline, hydroxy-proline, and lysine, as well as ornithine should have this effect. Lysine was the only one of this group used in these experiments but, in the amount given, it was not found to have any specific supplementary effect upon the heated lactalbumin. At the 9% level, and with the intakes noted, lactalbumin provided less than the amounts required for the growth of rats of lysine, histidine, valine, threonine, methionine (Rose, '37), and glutamic acid (Rose, Oesterling and Womack, '48), but adequate amounts of isoleucine, arginine, and leucine. The improved growth of the animals fed the unheated and heated diets supplemented with valine, threonine, and methionine is in accord with this consideration. However, histidine additions had only a depressive effect, and leucine and arginine both improved the growth value of the diets. According to recent studies (Melnick et al., '46), the body needs all of the essential amino acids at the same time. Cook, Fraenkel-Conrat, Singer and Morgan ('51), in paper III of this series, found that the amino acids lysine,

methionine, and tryptophan are rendered partially unavailable in heated casein and in the lactose-containing lactalbumin used in these experiments, presumably because of the inability of proteolytic enzymes to set them free during digestion. It would seem logical to suspect that the reason for the failure of any one amino acid to improve the heated protein is that other amino acids needed along with it for tissue synthesis have not been liberated, in digestion, in sufficient amounts.

TABLE 4

The protein efficiency of lactose-free and lactose-containing lactalbumins unheated or heated at 140°C. for 30 minutes (9 rats in each group)

KIND AND CONDITION OF LACTALBUMIN [1]	TOTAL WT. GAIN IN 28 DAYS	PROTEIN INTAKE	PROTEIN EFFICIENCY ± STANDARD DEVIATION	"t" VALUE [2]
	gm	*gm*		
A (soluble), unheated	59	17.2	3.43 ± 0.32	
A (soluble), heated	57	17.2	3.31 ± 0.34	0.76
A (35–1),[3] unheated	64	16.8	3.87 ± 0.48	
A (35–1), heated	44	17.9	2.48 ± 0.23	7.81
A (35–2), unheated	56	17.9	3.13 ± 0.38	
A (35–2), heated	55	17.9	3.11 ± 0.29	1.10
B (15–42),[3] unheated	58	16.9	3.42 ± 0.42	
B (15–42), heated	50	16.5	3.03 ± 0.24	2.40

[1] See table 1 for description of the lactalbumins.

[2] Comparison of the mean protein efficiency of the unheated lactalbumin with that of the heated lactalbumin of the same kind by the method of Snedecor ('46). For 16 degrees of freedom a "t" value of 2.120 at the 5% level is significant. A value of 2.921 is significant at the 1% level. Values higher than this are highly significant.

[3] Contained lactose; the others were lactose-free.

Later, when other samples of lactalbumin were made available, another series of experiments was carried out in which the lactalbumins described in table 1 were fed unheated and heated dry at 140°C. for 30 minutes. The results of these experiments are summarized in table 4. The unheated samples all promoted approximately equal growth; a significant decrease in protein efficiency as a result of heat was found only in the two lactalbumins which contained lactose.

It was obvious from these results that a significant decrease in the protein efficiency of lactalbumin, even at these high temperatures, ocurred only when the lactalbumin contained lactose. Since the exact method used to prepare the commercial lactalbumins was unknown, a small amount of ethanol-precipitated lactalbumin, which was lactose-free, was prepared as described in footnote 3, table 1. Four groups of rats were fed diets containing this lactose-free lactalbumin treated as follows: raw; heated at 150°C. for 70 minutes;

TABLE 5

The effect of lactose treatment or heat, or both, on the protein efficiency (p.e.) of a lactose-free (ethanol-precipitated) lactalbumin

CONDITION OF LACTALBUMIN	TOTAL [1] WT. GAIN	PROTEIN INTAKE	P.E. AND STANDARD DEVIATION	DECREASE IN P.E.	"t" VALUE [4]
	gm	*gm*		%	
Raw	35	14.0	2.47 ± 0.28	..	....
Heated [2]	30	12.0	2.49 ± 0.07	0	0.170
Lactose combined [3]	34	14.0	2.42 ± 0.24	2	0.225
Lactose combined, heated [2]	22	12.0	1.83 ± 0.44	26	2.960

[1] Six rats in each group were fed for 21 days.

[2] Heated at 150°C. for 70 minutes.

[3] Treated with lactose under the mild conditions.

[4] Comparison of the mean protein efficiency of the unheated lactalbumin with that of the heated or lactose combined samples or both, by the method of Snedecor ('46). For 10 degrees of freedom, the value for "t" of 2.960 falls between the 1% and 2% levels.

chemically combined with lactose by the mild method of treatment; and the latter heated at 150°C. for 70 minutes. Such large amounts of whey were required to produce this lactalbumin that it was impossible to prepare enough of the product to feed larger groups of animals and to continue them on experiment longer than 21 days. The results of this experiment may be seen in table 5. Even at this extremely high temperature (150°C.), the protein efficiency of the original lactose-free product was not appreciably altered nor was it changed significantly by mild lactose treatment in which between 2 and 3% of lactose was bound. However, when the

lactose-containing protein was heated at this temperature, the protein efficiency was decreased about one-fourth. No browning was observed in the lactose-free product after heating, but the one which contained lactose was changed to a deep caramel color.

Casein

In order to study the effect of heat on the nutritional value of casein before and after it was combined with lactose, rats were fed diets containing 12% protein supplied by casein untreated, and treated as follows: refluxed in water; refluxed in a lactose solution; and combined with lactose under mild conditions (52°C. for 24 hours). Each of these diets was fed both unheated and heated at 140°C. for 30 minutes and with and without the addition of 0.2% lysine. The results are reported in table 6.

All of the caseins which were subjected to heat or lactose treatment, or both, when compared with raw casein were found to have decreased protein efficiency. The degree of damage caused by dry heat, refluxing in water with and without additional heating, and combination with lactose under the mild conditions of treatment was of the same order. The caseins refluxed in a lactose solution and those combined with lactose by the mild conditions and then heated were decreased in protein value somewhat more than the others mentioned above. In both of the latter samples the protein damage could be attributed to the combined effects of lactose, water (which is required for chemical reaction between the protein and lactose), and heat. Water washing alone appeared to depress the nutritive value of the casein in a manner probably different from that of the Maillard effect, since addition of lysine did not improve the nutritive value and additional heating had no further effect. Also, the casein contained no detectable carbohydrate and any that might have been present, unless bound, would have been removed in the washing process.

The decreased protein value which resulted in the casein refluxed in water at 95°C. cannot be attributed entirely to the

TABLE 6

The effect of heat or lactose combinations, or both, and the addition of 0.2% L-lysine monohydrochloride on the protein efficiency (p.e.) of casein

TREATMENT OF CASEIN [1]	EFFECT OF HEAT AND/OR LACTOSE COMBINATION					EFFECT OF HEAT WHEN LYSINE WAS ADDED			
	Protein intake	Total weight gain [2]	P.e. and standard deviation	"t" value [5]		Protein intake	Total weight gain [2]	P.e. and standard deviation	"t" value [5]
				[6]	[7]				[8]
	gm	*gm*				*gm*	*gm*		
Untreated	28.5	72	2.53 ± 0.23			28.4	73	2.52 ± 0.16	
Same, heated	28.5	65	2.28 ± 0.18	2.381	2.381	28.6	69	2.44 ± 0.25	0.692
Reflux with water	28.9	66	2.28 ± 0.17		2.451	29.6	69	2.33 ± 0.17	
Same, heated	29.5	67	2.30 ± 0.24	0.193	2.127	29.5	61	2.05 ± 0.28	2.435
Lactose combined [3]	28.4	65	2.29 ± 0.27		1.920	28.4	71	2.51 ± 0.52	
Same, heated	29.1	63	2.16 ± 0.26	1.000	3.032	29.7	64	2.15 ± 0.17	1.846
Reflux with lactose [4]	28.4	62	2.18 ± 0.26		2.800	27.5	55	2.00 ± 0.28	
Same, heated	25.0	47	1.89 ± 0.21	2.417	5.311	28.0	56	2.01 ± 0.31	0.070

[1] The caseins, treated as indicated, were added to the diets in an amount to supply 12% of protein to the diet. All heating was carried on at 140°C. for 30 minutes.

[2] Average gain per rat for 8 rats in a group fed for 28 days.

[3] Reacted with lactose under the mild conditions (52°C. for 24 hrs.), in which 3 to 4% of lactose was combined.

[4] Contained 7–8% of bound lactose.

[5] The "t" values according to Snedecor ('46). At 14 degrees of freedom — 2(n—1) — a value of 2.145 is significant at the 5% level; a value of 2.977 is significant at the 1% level. Larger values of "t" are highly significant.

[6] Mean value compared with that of the similarly treated casein, unheated.

[7] Mean value of treated compared with that of the original untreated casein.

[8] Comparison of the mean protein efficiencies of the heated and unheated preparations, both supplemented with lysine.

effect of heat, since dry heat as high as 100°C. was found by Greaves, Morgan and Loveen ('38) not to have this effect. They found also that washing casein in cold water lowered the protein value for rat growth as much as did boiling it for two hours. Dry heat at temperatures above 100°C. (in this case, 140°C. for 30 minutes) affected the utilization of lysine, as shown by the improved growth value of the casein when lysine (0.2%) was added to the diet. This confirms the findings of Greaves, Morgan and Loveen that lysine is mainly involved when casein is heated at this temperature.

The improvement made in the growth value by the addition of lysine to diets containing casein combined with lactose under mild conditions (52°C. for 24 hours) gives evidence that the initial site of reaction between the protein and carbohydrate is mainly with the ε-amino group of the lysine. When the casein combined with lactose was subjected to additional heat treatment, there was significant decrease in the protein efficiency, which was not improved by the addition of lysine. This indicates other changes in the protein molecule — probably the involvement of amino acids in addition to lysine. Refluxing in a lactose solution produced a similar decrease in the protein value. Evidence presented in paper III in this series (Cook, Fraenkel-Conrat, Singer and Morgan, '51) seemed to show, in acetylation experiments, that the major site of reaction between lactose and casein was with the amino groups. On the basis of their findings and those of other workers cited in this report, these authors have reasoned that heat treatment, after the initial reaction between the lactose and ε-amino group of lysine, and also combination with lactose under the more strenuous conditions of refluxing, may cause formation of cross-links involving other amino acids in addition to lysine. The decreased *in vitro* digestibility and availability to microorganisms of the three amino acids measured — lysine, methionine, and tryptophan — also lend further support to this theory. Such linkages, if resistant to enzymatic hydrolysis, could account for the failure of lysine alone to restore the protein efficiency of these proteins.

Patton, Hill and Foreman ('48) found decreased amounts of lysine, arginine, and tryptophan in casein refluxed under similar conditions in a glucose solution. They did not, however, reflux the casein in water without the carbohydrate, as was done in this experiment. The decrease observed as a result of the reaction of casein with lactose under the mild conditions is in contrast to the finding that both lactalbumin combined with lactose under similar conditions, and commercially prepared lactalbumins containing lactose, do not have decreased protein efficiency (tables 4 and 5). With lactalbumin, rats appeared to be able to break the bonds formed between the protein and lactose; with casein, they did not. The result of further heat treatment of the lactose-containing lactalbumin was, however, comparable to the findings with casein, in that other amino acids appeared to be rendered unavailable.

The apparent depression in the protein efficiency of the casein refluxed with water and heated and that refluxed in a lactose solution when lysine was added is difficult to explain. Lysine in excess of 1% of the diet has been found by the present authors, with casein (unpublished results), to depress rat growth, and by Henry and Kon ('48), with powdered skim milk, to decrease the biological value of the protein. The latter workers concluded that it was not so much a matter of toxicity as of failure to utilize the excess amino acid.

SUMMARY

A significant decrease in the protein efficiency of lactalbumin for the growth of rats was produced by heat only in samples which contained lactose or which had been chemically reacted with lactose.

No change was found in the protein efficiency of a commercially-prepared lactalbumin containing lactose when heated dry at 100°C. for 30 minutes. But when the temperature was increased to 120°C. for the same period, the protein efficiency was decreased by 12%, and when the temperature was raised to 130°C. it was decreased by 19%.

Rats fed diets containing lactalbumin which had been heated at 130°C. for 30 minutes during the first 22 weeks of the experiment, and at 140°C. for 30 minutes during the remainder of the year, grew steadily throughout the period, but they did not attain the same average weights as those fed unheated lactalbumin, even though they had free access to food for the last 11 months.

The growth values of both heated and unheated lactalbumin were slightly improved by supplementing them with the amino acids valine, leucine, threonine, methionine, and arginine; lysine improved only the unheated protein. Histidine depressed the growth value of both the unheated and heated lactalbumin, and when combined with lysine depressed the value of the unheated protein. Only ornithine lessened the difference in growth values caused by heat treatment of lactalbumin. This is in contrast with previous findings that lysine and, to a lesser extent, histidine partially restored the growth value of heated casein.

The protein efficiency of casein was significantly decreased when it was refluxed with water or combined with lactose under the mild conditions of treatment. It was decreased slightly more when refluxed in a lactose solution, and about twice as much when the latter sample was heated. The addition of 0.2% lysine to mild, lactose-combined casein raised the protein efficiency to that of the raw casein; but after this product was heated, its protein value was not restored by the lysine. Addition of lysine did not affect the value of the other treated samples.

Three forms of change in the nutritive value of the casein appeared to be involved: that caused by water washing alone, apparently not involving lysine and not aggravated by heating; that caused by dry heat, and partially restored by lysine; and that caused by reaction with lactose under the mild conditions, and reparable by lysine. This last change was aggravated by additional heat, which caused decreased availability of other amino acids so that the damage was not corrected by lysine.

Combination between the protein and lactose under the mild conditions of treatment resulted in decreased protein efficiency of the casein, but not of the lactalbumin. Heat treatment, after lactose-combination, was equally deleterious to the quality of both proteins.

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DIETARY CONSTITUENTS WHICH MAY INFLUENCE THE USE OF FOOD CHOLESTEROL

I. EGGS: BIOTIN AND AVIDIN [1]

RUTH OKEY, R. PENCHARZ,[2] S. LEPKOVSKY AND ELEANOR R. VERNON
WITH THE TECHNICAL ASSISTANCE OF DOROTHY JEROME AND MONA MARQUETTE
Department of Home Economics and Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley

TWO FIGURES

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There is recent evidence, part of it more or less contradictory, that food cholesterol may influence either serum cholesterol concentration or the form of its distribution, and possibly have something to do with lipid precipitation in arteriosclerotic lesions (Gofman et al., '50; Keys, '50). The fact remains, however, that cholesterol-rich foods have been consumed in large amounts by many people, throughout long lifetimes, with no apparently bad results. It has seemed logical to investigate the effect of the substances accompanying cholesterol in foods on its retention in tissue.

The egg is, in terms both of concentration and amount consumed, our most important food source of cholesterol. One yolk may contain 0.23 to 0.30 gm. This is accompanied by protein of excellent quality, choline-containing phospholipid, vitamins A and D, and generous amounts of B complex including biotin and pantothenic acid. The white of the egg contributes extra protein and riboflavin but it also supplies,

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[2] Present address: Research Laboratory for Experimental Biology, Stanford Research Institute, Stanford, California.

in quantity, antitrypsin and the biotin antagonist, avidin. Biotin deficiency can be produced by ingestion of uncooked whole egg. Investigation of the properties of a food of such complex composition leads inevitably to problems involving simultaneous variation in more than one of its constituents.

The primary purpose of this paper is to present data showing the effects of biotin and avidin on food cholesterol retention in the rat. In order to secure comparable data and to rule out variations due to changes in such egg constituents as protein, fat and choline, it has been necessary to compare data from groups of animals fed as nearly uniform diets as possible. Five basal diets were used. They were fed alone and with the following variations: cholesterol added; biotin added; biotin and cholesterol added. Tabulated data are limited to representative groups of rats fed commercial dried egg products of good grade. Entirely consistent effects of biotin and avidin have, however, been noted in several hundred additional rats fed products varying from lyophilized egg white to dried egg rated as "unfit for human consumption."

Rats have the advantage, in dietary studies with eggs, of near immunity to the deleterious effects of the antitrypsin. They also have an equal immunity to arteriosclerosis. Absorbed cholesterol, in the normal rat, is rapidly esterified in the liver, where the ester remains in high concentration with little apparent damage to the liver cells (Okey, '41). Serum cholesterols, on the other hand, remain at comparatively low levels in cholesterol-fed rats. Total liver cholesterol has, therefore, been considered the best index of cholesterol retention in this species.

EXPERIMENTAL

A. Contrasts: Biotin-deficient and biotin-fed rats

Weanling rats of the Long-Evans strain were used for the work herein recorded. They were caged on wire screening and had access to water at all times. When intake was meas-

ured, food was weighed into non-scatter food cups and the uneaten food weighed back at least once weekly, when the animals were weighed and checked for deficiency symptoms. Except for two pair-fed series, the diets were given ad libitum.

At the end of an experiment the animals were anesthetized with sodium amytal, killed by bleeding from the heart, and subjected to careful autopsy. Livers were removed promptly, weighed, and samples taken for moisture and lipid determinations. Methods for cholesterol and fatty acid determination have been described elsewhere (Okey, '30, '46; Bloor, '28).

Diets

The composition of the diets used is given in table 1. In the first three pairs, I and VIII, II and III, IV and V, the protein content (about 20%) was adjusted to match that furnished when whole egg was used as a source of cholesterol. The "total" avidin of diets II and III matched that of the egg diet (I). However, the "high avidin" diets XIII and XIV had, as a result of the increased egg white, an increased protein content. An attempt was made to balance the methionine content of the "clearance" diets, X and XII, IV and V. The "fat" content of all the diets (13.5%) matched that of the whole egg diet I. Vitamins, except biotin, were added to all the diets in uniform amounts rated as adequate for normal growth when given with a vitamin-free diet. Diets were mixed in small quantities with a power mixer and refrigerated until fed. Biotin was sometimes given separately.

From the standpoint of adequacy the above diets may be classified as likely to produce: (a) mild biotin deficiency (I); (b) moderate deficiency (II and III); (c) severe deficiency (IV and V, XIII and XIV); and (d) adequacy achieved without a biotin supplement (X and XII). Avidin was apparently largely denatured during removal of cholesterol from the whole egg for diet VIII. Diets II and III were therefore sometimes fed with a partial biotin supplement (1.5 mg per rat per day), with which they produced a deficiency compar-

able to that observed for the whole egg diet, and gave the desired contrast between a cholesterol-rich and a cholesterol-free diet.

TABLE 1

Diets

(Parts per hundred)

DIET NO.	I	VIII	II	III	IV	V	X	XII	XIII	XIV
Dried whole egg	36.0									
Extracted whole egg		26.0								
Egg white, dry [1]			11.0	11.0	17.0	17.0			22.0	22.0
Casein (vitamin-free)	5.0	5.0	11.0	11.0	5.0	5.0	25.0	25.0	5.0	5.0
Fat (Primex)		13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5
Salts (Hubbel) [2]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sucrose	55.0	51.5	59.7	60.7	59.5	60.5	56.5	57.5	55.5	54.5
Cholesterol			1.0		1.0		1.0			1.0

Vitamin supplements per kg diet

Thiamine chloride	2 mg	Vitamin A	14,000 I.U.
Riboflavin	2 mg	Vitamin D	1,000 I.U.
Pyridoxine	2 mg	Mixed tocopherols	0.3 ml
Calcium pantothenate	10 mg		
Nicotinamide	10 mg		
p-Aminobenzoic acid	10 mg		
Folic acid	2 mg		
Choline	500 mg		
Inositol	500 mg		
Biotin, as indicated in various tables			

[1] On the basis of N content equivalent to approximately 80% protein.

[2] Hubbel, R. B., L. B. Mendel and A. J. Wakeman. A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273, 1937.

Food intake

Detailed discussion of the food intakes and growth rates of rats fed the whole egg diets, including those of the pair-fed series mentioned above, have already been published (Okey, Pencharz and Lepkovsky, '50). For the purposes of this paper the pertinent fact was that the animals continued to eat all the experimental diets during the development of biotin deficiency. In other words, food intake, while not so high for the animals on the unsupplemented diets as for those given biotin, was still sufficient when the diet contained cholesterol to account for a very considerable intake of this substance.

Figure 1 shows the effect on food intake and weight gain of two pairs of diets: I and VIII, which produced mild deficiency symptoms; and XIII and XIV, which rapidly produced severe deficiency symptoms. Biotin accounts for greater differences in the food intake of male rats than in that of females, hence this chart shows extreme variations.

There was very little difference in food intake between groups on the deficient diets and those on the paired biotin-supplemented diets until after the third week when, presumably, the biotin stored during lactation or from the mother's diet began to be exhausted. Rats fed whole egg continued to eat eight- to nine-tenths as much as their biotin-dosed controls, often 90 to 120 gm per week. After the third week, the growth rates of rats on the unsupplemented diets with 22% egg white fell sharply, but food intakes of at least 60 and 70 gm per week, for females and males respectively, were usually maintained to the 10th week.

For rats fed diets II and III with only 11.0% egg white, food intakes and growth rates resembled those for animals on diet I, although food intakes were usually 5 to 10 gm per week lower. Diets IV and V were used for biotin depletion in older rats only, and were still being consumed at average rates of 90 to 120 gm per week by both female and male rats when the experiments recorded in table 4 were terminated.

The casein diets, X with, and XII without cholesterol, were consumed, after 4 weeks, in amounts varying from 75 to 90 gm per week for females and 85 to 115 gm per week for males.

Gross symptoms of biotin deficiency varied to a considerable extent among rats fed the different deficient diets, and indeed among individual rats fed the same diet. Rats which

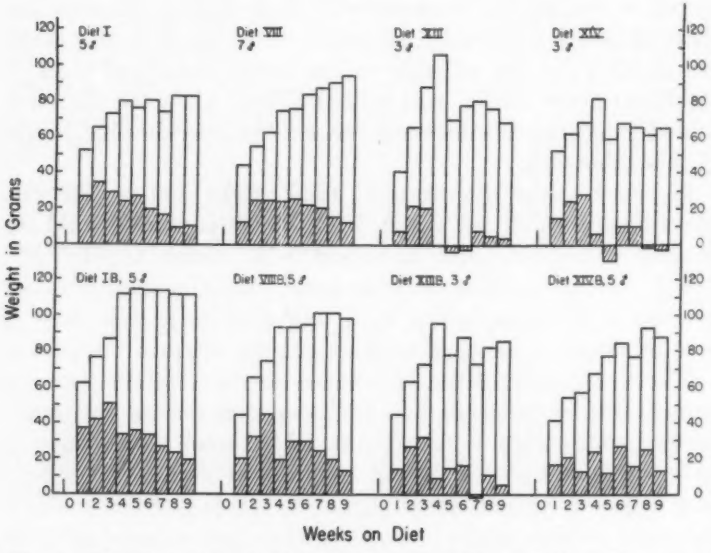

Fig. 1 The effect of ample biotin dosage (10 μg/10 gm diet) on the average food intake and growth rate of rats fed egg diets. Weight gains or losses are indicated by the shaded areas, food intakes by the total height of the bars above the base line. Biotin supplementation is indicated by the addition of the letter "B" to the number designating the diet.

grew rapidly during the first three weeks on the diet sometimes seemed to exhaust biotin stores and to show signs of depletion more rapidly than did litter mates that grew more slowly. It was definitely more difficult to get a typical deficiency in rats placed on diet when they weighed 55 gm than in those placed on diet at 45 gm. On the mildly deficient diets males showed earlier and more marked deficiency symptoms

than did females, but on the diets higher in egg white the sex difference tended to disappear. Figure 2 shows three typical male rats after 10 weeks on experimental diets of varying avidin content. Rats given adequate biotin with all the basal diets used were fine sleek animals with heavy and healthy coats.

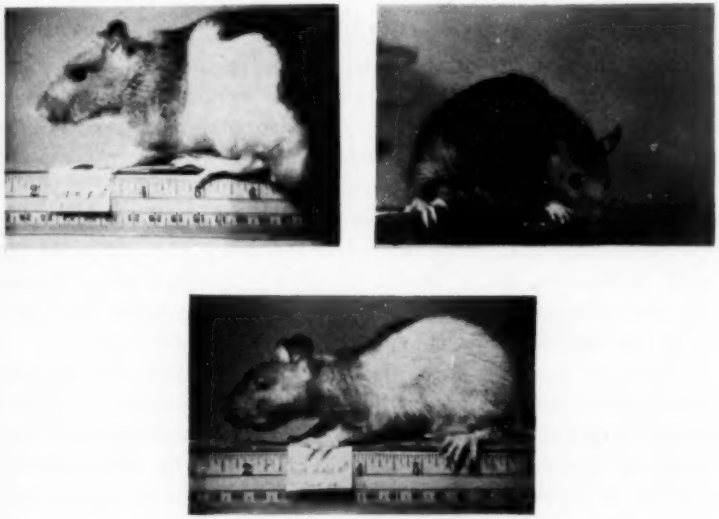

Fig. 2 Male rats after 10 weeks on diets containing different amounts of active avidin. Top left: rat fed diet VIII (extracted whole egg) shows few, if any, signs of deficiency. Top right: rat fed diet I (untreated whole egg) shows wrinkling of skin due to loss of subcutaneous fat, and partial alopecia, but no true dermatitis. This rat was still growing in length. Lower: rat fed diet XIV (22% egg white) shows beginning emaciation, unkempt appearance, hunched posture and dermatitis characteristic of severe biotin deficiency.

At autopsy, the lack of subcutaneous and visceral fat was the most striking characteristic of the deficient animals. Males lost fat earlier and more rapidly than did females when the diets of lower avidin content were given, but with the diets higher in avidin sex differences tended to disappear. Carcasses of rats showing only a mild alopecia, and with a

history of food intakes of approximately 85 gm for the week immediately preceding autopsy, were often almost free of visible fat. Stained sections of the skin of deficient rats showed thinning of the Malpighian layers and an almost complete absence of fat. Biotin-fed controls had, however, fully normal amounts of subcutaneous and visceral fat.

Livers

Gross and microscopic abnormalities in the liver were, on the other hand, observed only in the rats receiving biotin with the cholesterol-rich diets. These consisted of a moderate degree of enlargement and fatty infiltration. Liver sections contained material which stained red with Sudan IV. Livers were, however, neither as large nor as fatty as those of rats fed cholesterol with diets of higher fat and lower protein content. Moreover, while biotin-fed rats given the cholesterol-free diets sometimes had livers slightly larger than their deficient partners, they otherwise resembled them closely.

Chemical analyses showed that rats fed the biotin-supplemented diets had about the liver cholesterol values to be expected from the amount of protein, fat and cholesterol eaten when the diet was otherwise adequate (table 2). These were roughly 6 to 8 times as high for the cholesterol-fed rats as for their partners on the cholesterol-free diets. Fatty acid values seldom increased more than half that much.

Rats fed the biotin-deficient diets with cholesterol had total liver cholesterol values which usually decreased with time on diet until, at 10 weeks, they were likely to be as low as those of their normal controls fed cholesterol-free diets. This might occur when the rat was eating as much as 150 mg of food cholesterol daily. Liver fatty acids decreased at the same time but to a lesser degree (table 2). It was apparently necessary, however, for the rat to exhaust the biotin stored during lactation before it would entirely lose its ability to store liver cholesterol from a cholesterol-rich diet. Rats grossly resistant to the deficiency had higher liver cholesterol

values than those with more marked external deficiency symptoms. Females did, nevertheless, lose capacity to store excess liver cholesterol without showing the degree of alopecia seen in males.

TABLE 2

Effect of biotin on liver lipids in rats[1]

BASAL DIETS	BIOTIN SUPPLEMENT	NO. AND SEX OF RATS	AVERAGE WEIGHT AT END	LIVERS			
				Average weight	Fatty acid	Total cholesterol	
						Average	Range[2]
	mg/10 gm diet		*gm*	*gm*		*mg per liver*	
A: *With cholesterol*							
I							
Whole egg	0	17 ♂	183	7.8	417	53	21–141[3]
(low avidin)	5.0	15 ♂	312	13.8	1038	170	107–385
	10.0	10 ♂	314	12.0	962	190	87–301
II							
11% egg white	0	6 ♂	125	6.3	202	25	14–36
(moderate	0	7 ♀	108	4.7	188	22	16–31
avidin)	1.5	4 ♂	264	12.1	460	47	39–68
	1.5	4 ♀	214	8.3	305	32	28–37
	10.0	9 ♂	307	11.6	818	192	139–230
	10.0	3 ♀	207	8.9	522	172	165–177
XIV							
22% egg white	0	10 ♂	114	5.8	180	52	30–105[3]
(high avidin)	5.0	10 ♂	209	9.6	652	125	85–173
	10.0	10 ♂	226	9.5	640	124	93–177
B: *Without cholesterol*							
VIII							
Extracted egg	0	7 ♂	268	9.4	357	26	24–28
(very low avidin)	5.0	5 ♂	301	13.8	300	28	22–35
III							
11% egg white	1.5	4 ♂	322	11.5	391	24	21–27
(moderate avidin)	1.5	4 ♀	201	8.5	255	19	18–20
XIII							
22% egg white	0	10 ♂	147	5.5	186	16	13–26
(high avidin)	5	8 ♂	279	9.9	330	25	17–29

[1] Rats placed on diet at weaning, killed 9 to 10 weeks thereafter.

[2] Livers of two to 4 rats sometimes pooled for analysis. See text.

[3] High figures for rats with no obvious deficiency symptoms.

The "ranges" noted in the tables may require comment. At autopsy rats on the same diets were grouped by sex, similarity in size, history of food intake, external deficiency symptoms, and size, color and texture of liver. Liver samples from two to 4 rats were pooled accordingly, for lipid analysis. When a considerable number of discrepancies existed, livers were analyzed separately. This gives a reasonably good index

TABLE 3

Free and total liver cholesterol in representative groups of rats at different stages of biotin deficiency

BASAL DIETS	BIOTIN DOSE	NO. OF RATS [1]	DEFICIENCY SYMPTOMS	LIVERS				
				Average weight	Total cholesterol		Free cholesterol	
					Average	Range	Average	Range [2]
	mg/day			*gm*	*mg per liver*			
A: *With cholesterol*								
X								
No avidin (casein)	0	8	None	9.0	339	265–384	30	29–31
I								
Low avidin	3.5	8	None	8.5	208	141–324	26	22–30
(whole egg)	0	10	Moderate	7.8	58	24–74	21	16–25
II								
Moderate avidin	3.5	4	Very slight	8.0	113	96–130	24	22–26
(11% egg	1.5	4 ♂	Moderate	8.2	33	30–36	25	24–26
white)	0	4 ♂	Severe	6.7	14	11–16	14	10–15
B: *Without cholesterol*								
VII								
Very little avidin (extracted egg)	0	8	None	7.6	19	18–20	17.5	15–20
III								
Moderate avidin								
(11% egg	1.5	4 ♀	Moderate	8.5	19	18–20	17	16–18
white)	1.5	7 ♂	Moderate	9.1	22	15–27	21	13–27

[1] Equal numbers of males and females per group unless otherwise noted.

[2] Livers from two to 4 animals of like sex and degree of deficiency sometimes pooled for analysis.

of variability and makes possible the use of increased numbers of animals, but the data cannot be evaluated by the usual statistical methods.

Variability in the time required for liver lipid depletion in rats fed the biotin-deficient diets was considerable. As noted above, this was associated with differences in the speed with which the individual rats developed external deficiency symptoms. The size at which the animal was placed on the deficient diet and the rate at which he grew in the first few weeks thereafter seemed to have as much to do with speed of biotin depletion, and also with depletion of liver cholesterol, as did actual days on the deficient diet or the amount of avidin it contained. Also, in some rats which grew very slowly at first on diets I and II, synthesis of biotin by intestinal flora seemed to become established to such an extent during lactation that no biotin deficiency developed. These animals retained relatively high percentages of liver cholesterol and account for a considerable proportion of the variability in the figures presented.

It is to be noted that in every case nearly all of the decrease in liver cholesterol produced by adding avidin to a cholesterol-rich diet was in the ester fraction (table 3). Ester values for the seriously deficient animals approached zero, while fatty acid values seldom fell lower than 3%. On adequate cholesterol-free diets of similar fat and protein content the rat stored little or no esterified cholesterol in the liver, and fatty acid values approximated those shown for diet VIII (table 3).

B. Effect of feeding egg white to rats following a period of liver cholesterol storage

A series of 64 weanling rats was given diet X, containing casein and cholesterol, for 4 weeks. At the end of that time 14 were killed in order to determine how much liver cholesterol had been stored. The rest were divided into 4 groups and given, respectively: (a) a diet containing avidin but no cholesterol (V); (b) a diet without avidin or cholesterol (XII);

(c) a diet containing avidin and cholesterol (IV); and (d) the original casein cholesterol diet without avidin (X). Rats of the first two groups were autopsied and their livers analyzed about 8 weeks after transfer. The first symptoms of biotin deficiency appeared in the groups fed egg white at this time. Because these symptoms were extremely mild in the non-cholesterol groups and because the rats of the cholesterol-fed groups showed even greater resistance to the deficiency, the rats of groups (c) and (d) were kept on the diets for an additional 4 weeks, then autopsied and their livers analyzed.

As the figures in table 4 indicate, there was no really significant difference in liver cholesterol between the group transferred to egg white (a) and that transferred to the casein diet without cholesterol (b). The third group (c), given egg white with cholesterol, showed a significant depletion of liver cholesterol, which had taken place while the cholesterol intake had been maintained at a high level. The food intakes of the animals in this group were as great as those of the 4th group (d) kept on the casein-cholesterol diet. In this last group the liver cholesterol had increased significantly over that of the rats killed after 4 weeks on diet.

Since the liver cholesterols of both the groups (a and b) transferred to the cholesterol-free diets had reached nearly normal levels before the rats were sacrificed, this experiment was repeated in order to ascertain whether avidin had increased the rate of liver cholesterol depletion. Seventy-two slightly older rats were used. This time the rats were sacrificed in subgroups at two, three, 4 and 5 weeks after transfer from the adequate cholesterol-rich diet. Liver cholesterol values for the control rats were within the limits noted for the previous series (table 4). Females transferred to the cholesterol-free diets with and without avidin showed no significant differences in rates of liver cholesterol depletion. Males given avidin during the depletion period had liver cholesterols averaging from 10 to 20 mg lower than those of the groups without avidin. They required 4 to 5 weeks to come within the ranges noted for the males of groups (a) and

(b), respectively (table 4), while females depleted to the corresponding levels within three weeks. At the end of this experiment, 5 weeks after transfer, rats given the avidin-rich diet with cholesterol still had average liver cholesterol values about twice as high as those noted for the corresponding

TABLE 4

Effect of change from adequate high-cholesterol to biotin-deficient diets

DIET NOTES	NO. AND SEX OF RATS	AVERAGE WEIGHT AT AUTOPSY	WEEKS ON DIET	LIVERS			
				Average weight	Fatty acid	Total cholesterol	
						Average	Range [1]
		gm		*gm*	*mg per liver*		
Controls							
at time of	7 ♂	136	0	8.0	600	156	107–190
transfer (X)	7 ♀	128	0	6.9	510	132	111–202
Changed to diet with:							
(a) avidin,							
no cholesterol	8 ♂	269	7½	9.9	386	27	20–35
(V)	8 ♀	200	7½	7.7	239	16	15–17
(b) no avidin,							
no cholesterol	6 ♂	327	8	11.2	325	27	21–30
(XII)	6 ♀	209	8	7.9	316	21	18–27
(c) avidin							
and cholesterol	6 ♂	246	12	10.2	592	65	45–85
(IV)	6 ♀	218	12	8.2	402	55	26–76
(d) kept on cholesterol							
without avidin	4 ♂	325	12	14.1	1195	321	244–500
(X)	3 ♀	250	12	8.9	806	285	235–369

[1] Samples from two to three animals showing like gross symptoms of deficiency were sometimes pooled for analysis. See text. Low values for animals with the more obvious deficiency symptoms.

group (c) of the previous series at 12 weeks. These were about half those of controls without avidin.

Data from this series, as from the previous one, seemed to indicate that feeding egg white is more valuable as a preventive of cholesterol storage in the liver when the diet

contains cholesterol than as a means of increasing the speed of depletion of stores already present.

DISCUSSION

Evidence that intake of vitamins of the B complex may affect liver storage of ingested cholesterol in the rat has been available for some time. It was observed in this laboratory in 1933 (Okey, '33) that removal of the source of the B complex (yeast extract)[3] from the diet of cholesterol-fed rats 10 days before autopsy produced a marked diminution in liver fat and cholesterol. That this was not due primarily to the decreased food intake was indicated by another study which showed that limitation of food intake in rats given an adequate cholesterol-rich diet resulted in storage of almost as much cholesterol in the small livers of the rats on restricted food intake as in the enlarged livers of rats fed the same diet ad libitum (Gillum and Okey, '36). Later Engel ('41, '42) reported depletion of liver lipid as a result of pantothenic acid deficiency. A preliminary note concerning the effect of avidin was published from this laboratory in 1947 (Okey, '47).

Evidence that biotin may produce fatty livers, as presented by Gavin and McHenry ('41), was based very largely upon work with rats previously depleted of the B complex and then given diets so high in fat and low in protein as to be, in themselves, conducive to the accumulation of liver lipid. The basal diets used by Best et al. ('46) and by Handler ('45) were likewise such as to make probable maximum responses in the way of liver lipid storage.

When whole egg is used as a source of cholesterol in the diet, conditions are quite different. The 2.0 to 2.5% cholesterol which the egg furnishes, on the basis of dry weight, is accompanied by some 48% of methionine-rich protein, and there is nearly 2% of choline included with the lipid of the dry egg. In other words, it is impossible to make a diet high in

[3] Harris.

cholesterol by giving whole egg without at the same time rendering it high in at least two substances, protein and choline, which are rated as potent lipotropic agents.

The study here reported presents evidence which is of special interest, therefore, because the data indicate that biotin and avidin affect liver cholesterol storage when the basal diets are high in methionine-rich protein as well as in choline-containing phospholipid. Present therapeutic practices in the treatment of arteriosclerotic lesions often call for a high protein diet as well as a lipotropic agent. Perhaps the substances prescribed in the latter category need to be evaluated with regard to function when given with diets used therapeutically rather than with a low-protein high-fat regimen.

The comparative well-being of the otherwise adequately fed rat with a mild biotin deficiency, together with his ability to ingest a cholesterol-rich diet without excess storage of liver cholesterol ester, suggest that avidin may possibly be of use in an emergency to help to clear out tissue cholesterol deposits. Experimental data indicate, however, that its use in this connection would be of doubtful value.

Esterification and storage of cholesterol in the liver are normal responses to ingestion of a cholesterol-rich diet by the rat, and responses apparently dependent upon the availability of biotin. When biotin is lacking, esterified cholesterol disappears from the liver and, at the same time, there is a spectacular loss of subcutaneous and visceral fat. This may be taken to indicate that biotin is necessary primarily for some reaction resulting in the esterification, or even the formation and storage, of fatty acids, and only incidentally for the storage of cholesterol. However, with the addition of biotin to egg diets, storage of liver cholesterol ester became proportionately greater than that of glyceride. This suggests that, with such diets, there may be some priority in the use of fatty acid to esterify cholesterol in the liver. Evidence that the physiological defect in biotin deficiency is not one of fatty acid absorption has recently been reported by Woodruff ('50).

SUMMARY

Varying degrees of biotin deficiency were produced in young rats by feeding otherwise adequate "purified" diets made high in cholesterol by adding dried whole egg or by adding cholesterol, as such, with dried egg white. These diets furnished 20 to 25% protein and about 13.5% fat.

When supplemented with ample biotin, they produced, in control rats, liver fatty acid values one and one-half to three times, and total liver cholesterol values 6 to 8 times, as high as those of rats fed similar adequate cholesterol-free diets. Most of the increase in cholesterol was in the esterified fraction. Biotin-deficient rats fed the egg diets high in cholesterol failed to store excess liver cholesterol ester, even when they maintained nearly normal food intakes. Loss of subcutaneous and visceral fat occurred at the same time.

When rats were transferred from an adequate diet which had produced high liver cholesterol storage to an avidin-rich diet without cholesterol, it could not be clearly shown that the avidin increased the rate of liver lipid depletion. Transfer to an avidin-rich diet containing cholesterol did, however, result in a marked decrease in liver cholesterol ester, in spite of a continued high cholesterol intake. The possible relationship of biotin supply to the ability of the animal to esterify and store fatty acid as well as cholesterol is discussed.

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NUTRITIONAL IMPROVEMENT OF WHITE POLISHED RICE BY THE ADDITION OF LYSINE AND THREONINE

LOUIS J. PECORA AND JAMES M. HUNDLEY
National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Maryland

ONE FIGURE

(Received for publicaton October 19, 1950)

Rice is the most important staple cereal of the world since it is the basic daily diet of more than half the human race. In view of the current world shortage of rice, any means of its nutritional improvement would be important. Recently an unsuccessful attempt was made to produce a satisfactory growth response in rats on an unsupplemented 90% white polished rice diet (Pecora et al., '50). Supplementation with the most deficient essential amino acid, lysine, did not improve the nutritional value of polished rice beyond that of unsupplemented rice. It was decided to investigate further whether a 90% polished rice diet could in any way be improved by the addition of certain "limiting" essential amino acids. The many supplements of essential amino acids employed — singly or in groups of two, three or 4 — failed to improve the nutritional value of rice except for the combination of lysine and threonine. This combination gave a threefold increase in growth over that obtained with rice alone, but this was still a suboptimum response.

Similar suboptimum growth responses have been reported by other investigators, using different cereals or cereal proteins supplemented with amino acids. Kligler and Krehl

('50), Harris et al. ('43) using zein, and Albanese et al. ('49) using zein and corn gluten, have reported similar observations: that supplementation of cereal protein with the most limiting essential amino acid or acids produced only a limited improvement of the protein as determined physiologically by growth response in the young rat.

EXPERIMENTAL METHODS

White polished rice of commercial grade bought in the open market and ground to 40–60 mesh was used in all these experiments. Test diets were prepared so that the sum of the different essential amino acid supplements and the rice totalled 90%; fat, salt mixture and vitamins made up the rest. The diets were adequate in all respects except for the protein, which was supplied solely by the rice at approximately a 5% level. The 4 lots of rice used in these experiments contained 0.94, 1.06, 1.23, and 1.14% nitrogen. The stock diet was analyzed as containing 3.82% nitrogen. The amino acid composition of one lot of rice, as analyzed by microbiological methods,[1] was found to agree within the range of experimental error with the compiled data of Block and Bolling ('45). The other lots were not analyzed. Each essential amino acid was added to the experimental rice diets in the amount suggested by Rose ('37) for the growing rat, making appropriate allowance for inactive enantiomorphs. Thus, the essential amino acids supplied by the rice in the diets were in excess of the requirements defined by Rose. The diet composition and the amount of each amino acid added are presented in table 1. It is realized that Rose's figures on the minimum amount of each essential amino acid required by the growing rat may be accurate only under the conditions of his experiments, in which he used crystalline amino acids and liberal amounts of the non-essential amino acids. These data would not necessarily be quantitatively accurate when studying diets containing both intact protein

[1] We wish to express our appreciation to Mr. Howard A. Bakerman of this Institute for the microbiological assay of the amino acids in rice.

and crystalline amino acids and with the non-essential amino acids supplied only by the rice protein. Nevertheless, they were used in the present study as the best available guides for comparative purposes.

The order of deficiency of the essential amino acids in rice was calculated from its amino acid composition (Block and Bolling, '45) and the minimum amino acid requirements for the growing rat (Rose, '37). When calculated on this

TABLE 1

Composition of diets[1]

CONSTITUENTS	AMOUNT	VITAMIN POWDER: Each gram supplied	
	gm		*mg*
Ground white polished rice (40–60 mesh) and one or more of essential amino acids to total	90	Thiamine HCl	0.2
		Riboflavin	0.3
		Pyridoxine HCl	0.25
		Nicotinic acid	2.0
Cottonseed oil (Wesson)	3	Ca pantothenate	2.0
Cod liver oil	2	Choline Cl	100.0
Salt mixture (Osborne-Mendel, '17)	4	Inositol	10.0
		Menadione (vit. K)	0.1
Vitamin powder	1	Biotin	0.01
		Folic acid	0.1

[1] Alpha-tocopherol (3 mg) was given by supplement dish once a week. Vitamins A and D were supplied in the diet by cod liver oil, each gram of which supplied at least 850 and 85 U.S.P. XI units of vitamins A and D, respectively.

The quantities of respective amino acids used for supplementation, in grams per 100 gm of diet, were as follows: L-arginine-HCl 0.243, L-histidine-HCl 0.40, DL-lysine-HCl 2.0, DL-tryptophan 0.20, DL-phenylalanine 0.70, DL-methionine 0.60, DL-threonine 1.20, DL-leucine 1.80, DL-isoleucine 1.0, and DL-valine 1.40.

basis there is a deficiency of all the essential amino acids except arginine, which is present in approximately two-fold excess. The approximate amounts of the essential amino acids supplied by the rice at the level used in these experiments are listed in table 2. These values varied slightly in proportion to the protein content of the rice. It must be kept in mind that in all these supplementation experiments an excess of arginine was present. None of the non-essential amino acids was used for supplementation.

Also listed in table 2 is the relative proportion of each of the essential amino acids in rice protein when compared to a standard whole egg protein, as calculated by Mitchell and Block ('46). No attempt was made in the calculations presented in table 2 to allow for the well-known sparing action of niacin, cystine and tyrosine on tryptophan, methionine and phenylalanine, respectively. When these factors were considered in calculating the amounts of each amino acid supplied by the 90% rice, the deficit of phenylalanine

TABLE 2

Comparison of the essential amino acid composition of white rice with the minimum requirement for rat growth and also with the essential amino acids of whole egg proteins

AMINO ACIDS	REQUIREMENT FOR GROWTH [1]	SUPPLIED BY 90% RICE [2]	DEVIATION FROM GROWTH REQUIREMENT	DEVIATION FROM WHOLE EGG PROTEIN [3]
	% diet	*% diet*	%	%
Lysine	1.0	0.22	— 78	— 56
Histidine	0.4	0.10	— 75	— 29
Methionine	0.6	0.23	— 62	— 17
Tryptophan	0.2	0.09	— 55	— 13
Threonine	0.5	0.27	— 46	— 16
Phenylalanine	0.7	0.43	— 38	+ 6
Valine	0.7	0.44	— 37	— 14
Leucine	0.8	0.53	— 34	— 2
Isoleucine	0.5	0.35	— 30	— 34
Arginine	0.2	0.49	+ 145	+ 13

[1] Rose ('37).

[2] Calculated from compiled data, Block and Bolling ('45).

[3] Mitchell and Block ('46).

and tryptophan was sufficiently small to move them toward the bottom of the list when compared to Rose's suggested requirement. Since rice contains very little cystine, the percentage deficit of methionine would be altered only slightly. Then the order of deficiency of essential amino acids in rice would be lysine, histidine, methionine and threonine. After making simliar allowances in the comparison of rice with whole egg protein, the order of deficiency of the essential amino acids was still lysine, isoleucine, histidine, methionine

and threonine, as listed in table 2. It is to be noted that regardless of the method of calculation or the standard of comparison, lysine is still indicated to be the most limiting amino acid. The order of deficiency after lysine varied with the standard used for comparison. It should also be noted that threonine is only the 4th or 5th most deficient amino acid in these calculations.

Male litter-mate Sprague-Dawley (National Institutes of Health colony) rats about 24 days old, average weight 43 gm, were distributed equally into experimental groups. They were housed individually in elevated wide mesh wire-bottom cages and were fed the experimental diets and water ad libitum. All tests were continued for 21 days. Animals receiving a standard stock diet [2] were included in each phase of the experiment as an aid in establishing relative rates of growth.

RESULTS AND DISCUSSION

When the rice diet was supplemented with lysine, the calculated most limiting amino acid, growth response was no better than on an unsupplemented 90% rice diet (table 3). When the other deficient essential amino acids were added individually to the rice, growth response still was not improved over that obtained with the unsupplemented rice diet (table 3). This is not in agreement with the observations of Kik ('40), who reported a slight but statistically significant improvement of white rice when supplemented with lysine or methionine, although his experiments were conducted using conditions quite different from ours. When rice was supplemented with histidine, the calculated second most limiting amino acid, plus each of the remaining essential amino acids individually, growth was no better than with rice alone. Rice plus lysine supplemented with each of the remaining essential amino acids individually gave no better growth response than rice alone, except for the combination of lysine and threonine (table 3). This diet gave approximately a

[2] Purina Dog Chow.

threefold increase in growth. This growth response has been obtained consistently during this study, using different lots of rice at various times. This is approximately 42% of the growth obtained with a commercial stock diet.[3] The improvement of the nutritional value of rice by supplementation with lysine and threonine was highly significant statis-

TABLE 3

Average growth increments in grams per rat for three-week period when fed amino acid-supplemented rice diets

(Five rats were used for each group)

AMINO ACIDS ADDED	BASAL DIETS		
	Rice [1]	Rice and lysine [1]	Rice, lysine and threonine [1]
	gm	*gm*	*gm*
.....	....	6.6 ± 0.7	30.8 ± 2.2
Arginine	10.8 ± 4.4 [2]	7.0 ± 1.1	20.8 ± 3.1
Histidine	11.0 ±3.8	4.2 ± 1.7	29.0 ± 3.3
Lysine	9.0 ± 1.9	....	....
Tryptophan	7.2 ± 3.6	1.8 ± 3.1	31.6 ± 2.7
Phenylalanine	9.2 ± 2.2	3.4 ± 0.7	29.8 ± 3.9
Methionine	14.2 ± 4.0 [3]	3.8 ± 1.7	21.8 ± 3.8
Threonine	8.8 ± 2.2	28.0 ± 2.0	....
Leucine	6.2 ± 1.7	0.8 ± 1.7	19.2 ± 3.0
Isoleucine	6.0 ± 3.1 [4]	0.6 ± 1.7	22.4 ± 0.9
Valine	13.4 ± 3.6	0.4 ± 2.2	22.2 ± 3.1
Control rice diet with no supplement	12.0 ± 1.0 [3]	9.2 ± 2.3	7.2 ± 2.0

[1] The figures in each column represent an individual experiment with litter mates.

[2] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[3] One died.

[4] Two died.

tically. Figure 1 shows the averaged results of several experiments using rice alone (diet 2301) and rice plus lysine and threonine (diet 2624).

Table 4 lists the average food intake, the average body weight gained and the "nitrogen efficiency" of rats on a basal diet of rice and lysine supplemented with each of the essential amino acids individually. The term "nitrogen efficiency"

[3] See footnote 2, page 105.

is substituted for protein efficiency since both a natural intact protein and crystalline amino acids were fed simultaneously. It will be noted that rats receiving the rice, lysine and thre-

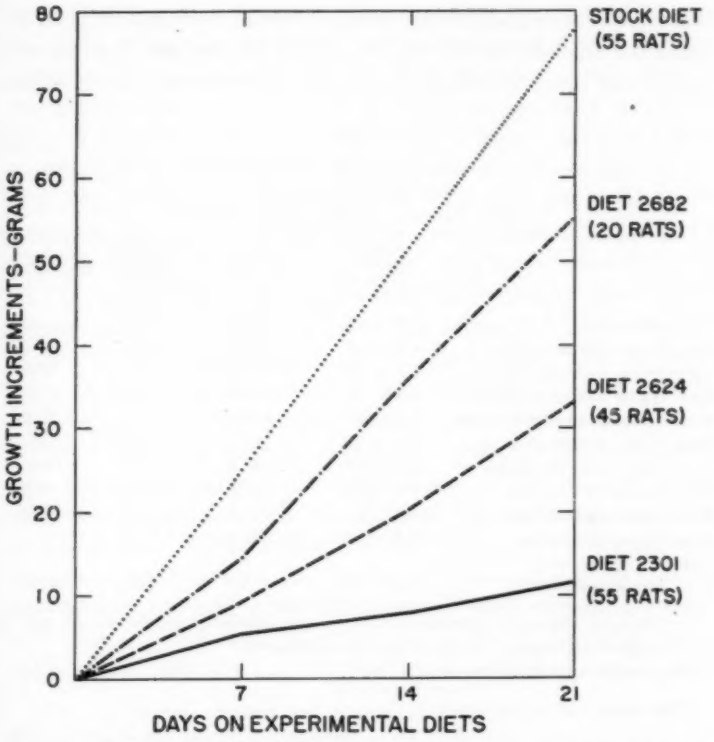

Fig. 1 Average growth curves of rats fed experimental diets. Average starting weight 43 gm. Diet 2301 = 90% rice; 2624 = rice, lysine and threonine; 2682 = rice plus all essential amino acids except arginine; stock diet = Purina Dog Chow.

onine diet gained 28.0 gm of body weight while consuming 142.8 gm of diet with a "nitrogen efficiency" of 14.43. On the other hand, rats on the unsupplemented rice diet gained only 9.2 gm while consuming almost as much diet (135.4 gm) with

a "nitrogen efficiency" of 6.17. On an absolute basis rats receiving the lysine- and threonine-supplemented diet consumed 0.45 gm more nitrogen than did rats on the unsupplemented rice diet. Even if this extra nitrogen were perfectly utilized to form additional tissue, it would be insufficient to account for the additional weight gained by the rats fed the supplemented diet. Furthermore, later experiments have shown

TABLE 4

Average growth increments and average food intakes in grams per rat for three-week period

(Five rats were used for each group)

DIET	AVERAGE GROWTH INCREMENT	FOOD INTAKE	N CONTENT OF FOOD INTAKE	NITROGEN EFFICIENCY [1]
	gm	*gm*	*gm*	
Rice and lysine	6.6 ± 0.7 [2]	121.2	1.56	4.23
Rice, lysine and histidine	4.2 ± 1.7	109.6	1.54	2.72
Rice, lysine and arginine	7.0 ± 1.1	122.4	1.65	4.24
Rice, lysine and tryptophan	1.8 ± 3.1	109.6	1.45	1.24
Rice, lysine and phenylalanine	3.4 ± 0.7 [3]	106.7	1.44	2.36
Rice, lysine and methionine	3.8 ± 1.7	114.2	1.54	2.47
Rice, lysine and threonine	28.0 ± 2.0	142.8	1.94	14.43
Rice, lysine and leucine	0.8 ± 1.7	102.8	1.43	0.56
Rice, lysine and isoleucine	0.6 ± 1.7	101.8	1.36	0.44
Rice, lysine and valine	— 0.4 ± 2.2	102.8	1.41	...
Control rice diet with no supplement	9.2 ± 2.3	135.4	1.49	6.17

[1] "Nitrogen efficiency" substituted for protein efficiency represents the gain in body weight in grams per gram of nitrogen consumed.

[2] Standard error of the mean, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[3] One died.

that the amounts of lysine and threonine used as supplements were considerably in excess of the minimum necessary to produce maximum growth stimulation. When the lysine and threonine supplements were reduced to one-fifth the amount used in diet 2624, thus reducing the nitrogen intake, growth was still equal to that obtained with full supplements. These facts indicate that the lysine-threonine supplements not only allowed the rats to consume somewhat more diet, but improved

the efficiency with which they utilized the limited amount of nitrogen in the diet. It is also clear that the improved growth with lysine and threonine cannot be entirely a matter of increased nitrogen concentration, since supplementation with lysine and essential amino acids other than threonine to yield a similar nitrogen concentration did not improve growth. It is evident from the data in table 4 that some of the amino acid supplements not only reduced food intake but also decreased "nitrogen efficiency." The reason for these results has not been determined.

Further attempts were made to improve diet 2624 by the addition of other essential amino acids, individually and in groups of two, three and 4. Rice supplemented with lysine and threonine (diet 2624) plus each of the remaining essential amino acids individually gave no improvement in growth (table 3). Supplementation of the rice, lysine and threonine diet (2624) with all possible combinations of pairs of the remaining essential amino acids, except arginine, gave a slightly inferior growth response, although the difference was not statistically significant. Many of the numerous possible combinations of three and 4 of the remaining essential amino acids[4] were added to diet 2624 without improving growth significantly. In no case, however, were growth responses obtained which approached that resulting from a rice diet supplemented with all the deficient essential amino acids, diet 2682 (N = 1.73 gm/100 gm of diet), which gave

[4] The combinations of three and 4 essential amino acids that were added to the rice, lysine and threonine diet were:

methionine, histidine, leucine
methionine, histidine, tryptophan
methionine, histidine, valine
methionine, histidine, isoleucine
methionine, histidine, phenylalanine
methionine, phenylalanine, leucine
methionine, phenylalanine, isoleucine
methionine, phenylalanine, histidine
methionine, phenylalanine, tryptophan
methionine, tryptophan, leucine
methionine, tryptophan, isoleucine
methionine, tryptophan, valine
histidine, phenylalanine, methionine, leucine
histidine, phenylalanine, methionine, isoleucine
histidine, phenylalanine, tryptophan, valine
histidine, phenylalanine, tryptophan, isoleucine

approximately 73% as much growth as was obtained with a stock diet (fig. 1).

A different approach was made in an attempt to clarify the unfavorable growth responses of the different diets. Diets were constructed so that they were supplemented with all the deficient essential amino acids but one, so that a different essential amino acid would be lacking in each diet. Diet 2682, which contained rice supplemented with all the essential amino acids except arginine, gave the best average growth response of 57.4 gm per rat for three weeks (fig. 1). The growth obtained with this diet was still not optimum, however, by comparison with that obtained with a stock ration. Growth response in all other instances was far below that obtained with the unsupplemented rice diet 2301. Surprisingly enough, the average body weight of the animals fed the diets unsupplemented with valine or leucine had fallen below their original starting weight at the end of the three-week period.

When all the essential amino acids were added at the level suggested by Rose ('37), the arginine content, already twice the required level, then became three times the minimum required level for the rat. Growth response with this diet was only 37.0 gm per rat for the three-week period, in comparison with the optimum growth response of 57.4 gm per rat for three weeks with diet 2682, which contained only that arginine naturally present in rice. When all the other essential amino acids were added at a threefold level individually, only methionine produced a significant depression in growth (32.0 gm growth per rat for three weeks). An excess of leucine or valine increased the growth response slightly, though the difference was not statistically significant, and the remainder of the amino acids resulted in no change from the optimum response with diet 2682.

The results of this study indicate that the nutritional value of rice is improved by supplementation with lysine and threonine but cannot be further improved by supplementation with other essential amino acids unless all are added together. Kligler and Krehl ('50) also observed that supple-

mentation of a 25% zein diet with essential amino acids in groups of three, 4, 5 and 6 produced markedly suboptimum growth in most instances. In no case could they equal the growth response obtained with 25% casein. Geiger ('47) supplemented zein and Harris et al. ('43) supplemented zein and also gliadin with essential amino acids and obtained some improvement in growth, though it was still suboptimum when compared to that obtained with casein. Albanese et al. ('49) reported a similar inferior growth response when they fed human infants synthetic diets made up of tryptopan- and lysine-supplemented zein or corn gluten. The data from the present study and the findings of several other investigators indicate that an amino acid that is calculated to be the most deficient or "limiting" is not necessarily the most deficient physiologically. The methods used in the present study to calculate the order of deficiency of essential amino acids predicted the fact that lysine was a limiting amino acid but failed to predict that threonine was equally limiting.

SUMMARY

Highly significant improvement of natural rice protein as it existed in polished rice was accomplished by the addition of two essential amino acids, lysine and threonine. This combination produced a growth response in white rats three times that obtained with an unsupplemented rice diet. Neither lysine nor threonine, nor any other essential amino acid, added to rice individually produced better growth than rice alone.

Rice plus lysine and threonine supplemented with all the other essential amino acids individually and with all the possible combinations of pairs was not further improved. Supplementation with many of the possible combinations of three and 4 of the remaining essential amino acids was without favorable effect. When rice was supplemented with all the deficient essential amino acids simultaneously, growth was improved considerably more than by supplements of only lysine and threonine but was still not optimum. These

results indicate that lysine and threonine are the most deficient essential amino acids in rice and that they are about equally limiting for rat growth.

A threefold excess of arginine or methionine when added to rice completely supplemented with all other essential amino acids produced a significant growth depression. An excess of the other essential amino acids individually was without effect.

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THE EFFECT OF PURIFIED DIETS UPON RUMEN FLORA

L. S. GALL,[1] W. E. THOMAS,[2] J. K. LOOSLI AND C. N. HUHTANEN[3]
National Dairy Research Laboratories, Inc., Oakdale, New York, and Departments of Animal Husbandry and Biochemistry and Nutrition, Cornell University, Ithaca, New York

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Previous reports (Gall et al., '49b, d) have shown that the composition of the ration has a marked effect upon both the types and numbers of rumen microorganisms. These changes in microorganisms can be related to differences in the digestibility of the ration and in the growth responses of the ruminants. It has been demonstrated that when sheep are fed a purified diet in which urea is the only source of added nitrogen, the 10 amino acids essential for rat growth are synthesized by rumen microorganisms (Loosli et al., '49). Bacteriological studies made on these same animals indicated that marked variations in rumen microorganisms were also found when different purified diets were fed. The present paper reports experiments in which the effect of specific ingredients in the purified diet exert marked effects upon rumen microorganisms and the growth responses of sheep.

EXPERIMENTAL

Two experiments have been conducted in which sheep were fed a purified diet consisting of cornstarch 42, corn sugar 25, cellophane 20, minerals 5, lard 4 and urea 4%. In the first

[1] Address: National Dairy Research Laboratories, Inc., Oakdale, New York.
[2] Present address: Department of Animal Industry, University of Kentucky, Lexington.
[3] See footnote 1.

experiment, 12 lambs were divided into three groups. Group 1 received the basal diet listed above; group 2 was fed the same diet except that sulphur was removed from the mineral mixture; and in group 3 the urea and part of the cornstarch were replaced with casein to furnish 10% of dietary protein. In experiment 2 the basal diet was changed to include 10% wheat straw in place of 10% cellophane. In both experiments the animals were fed vitamins A, D and E and choline, but no other vitamins were supplied. The details of these experiments pertaining to nitrogen and sulphur balances, growth and wool studies are being reported separately by Thomas et al. ('51).

Stomach tube samples of rumen contents from these animals were tested bacteriologically at various intervals while the animals were being fed the purified rations described above. The bacteriological studies consisted of an examination of the kinds and numbers of bacteria present in the rumen contents, using the following techniques: (1) a bacteriological slide count; (2) a Gram stain of the original rumen contents; and (3) an anaerobic cultural series prepared from high dilutions of the rumen contents. These procedures have been described by Gall et al. ('47, '49a). The cultures isolated from the animals on the purified rations were subjected to physiological studies in an effort to determine the general types of organisms present.

RESULTS

The pertinent data regarding the numbers of animals, rates of gain and general condition of the animals are summarized in table 1. On the basis of these data it is clear that while lambs made some growth on the purified diet containing urea and sulphur, the omission of inorganic sulfates resulted in complete failure of the animals to grow. Casein was not as effectively utilized as was urea.

Bacteriological data

The bacteriological findings on the three groups of animals in experiments 1 and 2, as summarized in table 2, showed

that the ration had a pronounced effect on both the kinds and numbers of the bacteria present in the rumen. The numbers of organisms present in the rumen, as determined by the total slide count, showed that the urea-plus-sulphur ration and the casein ration supported a bacterial population of about double that found in the rumen contents of animals fed the urea-without-sulphur ration. In the first experiment the animals in groups 1 and 3 averaged about 60 billion organisms per gram of fresh rumen contents, while the animals in group 2 had only about 33 billion. In experiment 2 the animals in

TABLE 1

The responses of animals fed purified diets

	DIET		
	Urea plus sulfur	Urea no sulfur	Casein
Experiment 1			
Number of lambs	4	4	4
Average daily gain (lb.)	+ 0.08	— 0.18	0.0
Condition of animals	good	very poor	poor
Number of deaths	0	3	1
Experiment 2			
Number of lambs	8	4	4
Average daily gain (lb.)	+ 0.19	— 0.12	+ 0.08
Condition of animals	good	very poor	fair
Number of deaths	0	1	0

groups 1 and 3 showed about 45 billion organisms per gram, but the animals on the urea-without-sulphur ration showed only about 25 billion per gram.

The morphological types of organisms present in animals on the three rations were also quite different, as was shown by the Gram stain of the original rumen contents. In both experiments 1 and 2, the animals fed urea-plus-sulphur showed Gram positive slender, curved rods or Gram positive large, fat rods as the predominating organism. This was in direct contrast to the animals on the urea-without-sulphur ration, in which either Gram negative medium rods or Gram

TABLE 2

Results of rumen bacteriological studies on sheep fed purified rations

	EXPERIMENT 1						EXPERIMENT 2		
	Test I	80 Days		Test II	150 Days		Test I	70 Days	
Group	1	2	3	1	2	3	1	2	3
Ration	Urea + S	Urea — S	Casein	Urea + S	Urea — S	Casein	Urea + S	Urea — S	Casein
No. of animals	4	4	4	4	2	3	8 (+ 1 repeat)	4	4
Predominating flora on Gram stain	Gram + slender curved rods; very large cocci	Gram — medium rods	Gram + cocci in masses	Gram + slender curved rods; lg. Gram + cocci	Gram — long slender rods in chains	Gram + cocci in masses	Med. Gram + lg. fat rods; med. cocci in pairs	Gram + fat rods divided in middle	Gram + cocci in masses
Ave. bacterial slide count (billion/gm)	60.3 (51.1–79.3)	33.4 (18.2–43.3)	65.5 (46.8–91.9)	64.3 (57.7–70.7)	25.6 (16.4–34.7)	61.4 (69.1–48.1)	46.1 (30.2–60.7)	25.3 (18.3–27.8)	43.5 (36.1–44.9)
No. of cultures:									
Total grown	16	12	13	17	6	13	29	16	9
Forming gas	8	2	13	6	0	8	9	2	4
Delayed growth	0	1	1	0	2	6	3	0	0
Clumping cotton	4	2	1	6	2	4	3	0	1
pH below 5.0	13	8	2	9	3	3	22	15	6
pH 6.0 or above	3	4	11	8	3	10	7	1	3
Anaerobes	16	2	13	15	3	10	25	3	9
Facultative anaerobes	0	10	0	2	3	3	4	13	0
No. of cultures similar to type culture:									
RO-575	15	0	0	14	1	4	12	3	4
RO-6TBR	0	0	0	1	0	0	6	0	1
RO-C8	6	1	6	3	0	4	2	0	3
RO-H_1	0	0	0	0	2	5	0	0	0
RO-Sp_1	0	0	12	0	0	0	0	0	0
RO-T_1	0	0	0	0	0	0	7	0	0
Facultative anaerobes	0	10	0	2	3	3	4	13	0

positive fat rods with a division in the middle were the predominating organisms. The Gram stains from the animals on the casein ration in both experiments 1 and 2 showed Gram positive cocci in masses as the predominating organisms.

The physiological characteristics of the organisms isolated from the animals in the three groups were also quite different. The culturability of the organisms was about the same on all three rations, but the types of organisms isolated were not. In table 2 a summary of the characteristics of these organisms, including gas formation, time of growth, clumping of cotton, final pH and degree of anaerobiosis, is listed for both experiments 1 and 2. The animals on experiment 1 were tested 80 and 150 days after being placed on the ration, while the animals in experiment 2 were tested after 70 days on the ration.

In all three tests, several characteristic physiological differerences were noted in the bacteria isolated from the rumen of sheep fed the three purified rations. The bacteria isolated from the sheep fed rations containing urea-plus-sulphur and casein were almost all obligate anaerobes. This is in marked contrast to the observation that most of the bacteria found in the rumen of sheep fed the urea-without-sulphur ration were facultative anaerobes. More gas-forming bacteria were isolated from the sheep fed the urea-without-sulphur ration. The majority of the bacteria isolated from the sheep fed urea produced a low pH *in vitro,* while the casein-fed animals tended to support a flora which did not lower the pH quite as much. This was most marked in experiment 1. Delayed growth did not usually occur on these purified rations, although in one test on the casein-fed sheep about half of the cultures showed delayed growth. Only a few cultures showing a clumping of the cotton were found, and some of these occurred on all rations.

An attempt was made in table 2 to plot the frequency of the occurrence of certain "type cultures" of rumen organisms. One type of culture, similar to the RO-575, occurred most frequently in the rumen contents of the animals fed

urea-plus-sulphur. In the first trial of experiment 1, 15 out of 16 cultures isolated from the urea-plus-sulphur-fed animals showed this type of organism, while none of the cultures from the other two groups showed a similar type of organism. In the second test of the first experiment, 14 out of the 17 cultures from the urea-plus-sulphur-fed animals were similar to RO-575, whereas only 4 of the cultures from the casein-fed animals and only one culture from the urea-without-sulphur animals were similar to this type of organism. Similar results were found in experiment 2.

Organisms classified only as facultative anaerobes were found as the predominating flora in the rumen of sheep fed the urea-without-sulphur ration. The organisms RO-Sp_1 and RO-H_1 were isolated in considerable numbers from the rumen of animals fed casein in the first and second tests, respectively, of experiment 1. RO-T_1 was isolated in fairly large numbers from the rumen of two of the poorer animals fed the urea-plus-sulphur ration. RO-6TBR and RO-C8 occurred sporadically on the urea-plus-sulphur and casein rations during all three tests. A description of some of the physiological characteristics of all of these organisms except RO-Sp_1 has been presented (Gall et al., '49c, '50). In the abstract, RO-T_1 was designated as N-109-8 and RO-H_1 as C-60-9 B1. RO-6TBR, RO-T_1 and RO-575 (now RO-L_1) will be further described in a paper now in press (Gall and Huhtanen, '51). The other organisms mentioned in the present paper will be described in papers soon to be prepared.

DISCUSSION

A comparison of the bacteriological picture found in the rumen of animals fed purified rations as opposed to that of animals fed the usual practical farm ration of hay and grain showed quite clear-cut differences. It is necessary only to examine the Gram stains of rumen contents taken from animals on these two types of ration to see this very striking difference. A description of the typical rumen flora found on practical farm rations has been presented in a previous publication

by Gall et al. ('49b). The Gram stains employed in this earlier work showed that a great variety of organisms were present in the rumen of these animals and that the fibers were heavily parasitized with bacteria. There were also large numbers of organisms free in the liquid. The picture seen on the Gram stain of rumen contents from animals fed a purified ration was much simpler. There were only a few types of organisms present, and because of the lack of fiber in the ration most of the organisms were lying free in the liquid. The bacterial slide counts from the rumen contents of the animals being fed a purified ration were similar to those found in animals fed a practical winter ration, but the types of organisms found in the cultures of the animals fed these two rations were quite different. The typical pattern obtained in the anaerobic cultural series from the rumen contents of animals on practical farm rations consisted of fast-growing organisms in the lower dilutions, and after these had been diluted out, the slower-growing organisms appeared in the higher dilutions. The slower-growing bacteria usually clumped the cotton and did not produce a low pH. In contrast, the rumen flora in the cultures from the animals on a purified ration consisted almost entirely of fast-growing organisms, which produced a low pH and seldom clumped the cotton. The types of cultures obtained from these animals more nearly resembled those found in the rumen of young calves which were being fed relatively large amounts of grain. The organism RO-575, which was prevalent on the urea-plus-sulphur ration and was found sporadically on the other rations, is seldom found in adult rumen but occurs frequently in the rumen of young calves (unpublished data). RO-T_1 is an organism which has seldom been isolated from adult rumen but is present in the rumen of young calves that are not in thrifty condition. RO-C8 has been isolated frequently from animals fed semi-purified rations (Burroughs et al., '50) and occurs occasionally in calves and adult ruminants on practical rations. The other organisms referred to in table 2 are frequently isolated from the rumen of calves and adult cows fed practical rations.

It is interesting to note that in addition to marked differences between the rumen flora found in animals fed practical and purified rations there were also clear-cut differences in the rumen flora found in animals fed several purified rations. A comparison of the rumen flora found in animals fed a ration containing urea-plus-sulphur or urea-without-sulphur showed striking differences. As might be expected, the omission of the one element, sulphur, produced a profound change in the physical condition of these animals, as the sheep fed the urea-plus-sulphur ration were thrifty animals, while the sheep fed the ration without sulphur were in an extremely poor physical condition and finally died. The rumen flora found in the animals fed the urea-plus-sulphur ration consisted chiefly of fast-growing obligate anaerobic organisms which were present in the amount of about 50 billion per gram. In direct contrast, when the sulphur was removed from this ration the rumen flora isolated from the sheep consisted of facultative anaerobes present in the amount of about 25 billion per gram. This is a drastic difference, since it implies a change in the whole mode of metabolism of food brought about through the agency of the bacteria in the rumen, and it is the only example in our experience of the isolation of facultative anaerobes as the predominating organism in rumen.

Differences in the rumen flora were also noted between the animals fed urea-plus-sulphur and those fed casein. This was interesting in view of the observation that the animals fed urea-plus-sulphur were in general in better physical condition than those fed casein. The numbers of rumen organisms found were in both instances about the same, but both the Gram stain and the physiological characteristics of the cultures isolated showed that these two rations supported different rumen flora. In the sheep fed urea-plus-sulphur the predominating organism isolated was a rod, RO-575. This organism was found only sporadically in animals on the other two rations. The RO-575 isolated from the animals fed the urea-plus-sulphur is able to synthesize several of the members of the B vitamins.

In the second experiment, organism RO-T_1 was present in two of the sheep fed urea-plus-sulphur. These animals were not in as good condition as the other animals in the same group. This organism does not synthesize any of the B vitamins and requires several members of the B complex vitamins for growth.

Another organism, RO-C8, was found in animals on the urea-plus-sulphur and casein ration, but was isolated only once from the sheep fed urea-without-sulphur. This organism is capable of producing several of the B vitamins. It is interesting to find that animals in better physical condition support rumen organisms which are capable of synthesizing rather than using some of the B vitamins. However, much further work must be carried out before there is true understanding of the interrelation between the physiology of rumen organisms and the physical condition of animals.

SUMMARY

1. Sheep fed three different purified rations, containing urea-plus-sulphur, urea-without-sulphur or casein, gave different physical responses as shown by their different growth rates.

2. The sheep on these three rations also supported different rumen flora, as was shown by the results obtained from studying the kinds and numbers of bacteria present in the rumen.

3. A lowered bacterial population was found in the sheep fed the urea-without-sulphur ration when compared with animals fed the urea-plus-sulphur or casein rations.

4. The sheep fed the urea-without-sulphur ration supported a rumen bacterial population consisting almost entirely of facultative anaerobes in place of the obligate anaerobes usually found in rumen.

5. Different physiological types of bacteria were found in the rumen of animals fed casein than in animals fed the other purified rations.

6. The rumen bacterial picture of the sheep fed purified diets was more like that seen in calves than in adults fed normal rations of hay and grain.

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NITROGEN REQUIREMENT OF NORMAL MEN ON A DIET OF PROTEIN HYDROLYSATE ENRICHED WITH THE LIMITING ESSENTIAL AMINO ACIDS [1]

WILLIAM S. HOFFMAN AND GORDON C. McNEIL

The Hektoen Institute for Medical Research of the Cook County Hospital and the Department of Physiology, University of Illinois College of Medicine, Chicago

SEVEN FIGURES

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It is now generally recognized that it is possible to obtain positive nitrogen balance in human subjects maintained on intravenously injected protein hydrolysates as the only source of nitrogen. However, the extent of that balance has been shown to be limited (Hoffman et al., '48; Kozoll et al., '45). Usually not more than 5 gm of daily nitrogen retention can be achieved, and even this accomplishment cannot be sustained for more than two or three weeks. Though this relative inefficiency of parenteral alimentation is partly due to technical factors, it is obvious that the efficiency would be enhanced if the biological value of the protein product were as high as possible. Even when hydrolysates are prepared from good proteins such as casein, the relative amounts of essential amino acids in the mixture are different from those of an ideal mixture of amino acids based on the true minimum requirements of the individual amino acids. If such a hydrolysate could be supplemented with the limiting amino acids in pure form, the requirement for nitrogen equilibrium of this

[1] These studies were aided by a grant from the Interchemical Corporation, New York, New York.

supplemented product should be considerably less than that of the original product, and its nutritional value correspondingly higher. Through the courtesy and help of Dr. W. C. Rose, we have been able to plan and carry out a nitrogen balance experiment on human subjects to determine the validity of this idea.

Dr. Rose made available to us his as yet unpublished findings [2] on the minimum requirements of the adult human male for the essential amino acids. When the pattern of these minimum requirements was compared with the quantitative pattern of essential amino acids in a modified lyophilized casein hydrolysate [3] which had previously been found to be clinically effective (Hoffman et al., '48), it was found that only tryptophan, phenylalanine, and methionine were present in relatively inadequate amounts in the casein hydrolysate. A new product was now prepared in which the casein hydrolysate was supplemented with the required quantity of these three limiting amino acids. Nitrogen balance experiments were then carried out in 7 normal human volunteers (male medical students ranging in age from 22 to 25 years) who, over a period of some 7 weeks, ingested as their only significant source of nitrogen a solution of the lyophilized hydrolysate with or without the supplementing amino acids. This report contains the essential findings in these experiments.

METHODS

The plan of the study was to place the group of volunteers first on a diet containing more than enough unsupplemented hydrolysate to produce nitrogen equilibrium, then to reduce the intake of nitrogen stepwise until negative nitrogen bal-

[2] Since the onset of this study preliminary reports of Rose's findings have been published (Rose, '47, '49).

[3] Elamine, I.C.-lyophilized, an acid hydrolysate of casein, from which about 50% of the glutamic acid and aspartic acid have been removed and to which DL-tryptophan has been added to replace that destroyed during the hydrolysis. This product (which is hereafter spoken of as the unsupplemented hydrolysate), and also the supplemented hydrolysate, were kindly furnished us by the Interchemical Corporation, Biochemical Division, Union, New Jersey.

ance was achieved, and then to raise the intake again until positive balance was again achieved. Thereafter the subjects were placed on a diet of the supplemented product considered sufficient to produce equilibrium. This quality proved too low and had to be raised in several steps until positive balance could be attained. The periods varied from three to 8 days. The plan of experiment kept the subjects from developing protein deficiency during the long period of study except for the short interval of negative balance with the supplemented product. It was believed that the total period of negative balance was too short and too insignificant to change the requirements for nitrogen equilibrium.

The basic non-protein diet consisted of a high carbohydrate drink made up of cartose [4] and a beverage base; soup made of carbohydrate, fat, and a nitrogen-poor vegetable; and muffins containing only a trace of nitrogen. A salt mixture containing the required minerals was specially prepared according to the method of Rose et al. ('50) and utilized in the prepared foods in the quantity of 4.5 gm daily. Sodium chloride ad libitum was allowed in the soup. Vitamins were provided in the form of one capsule daily.[5] The detailed daily menus and recipes are shown in the appendix. Macro-Kjeldahl analysis of the total daily basic diet showed an average nitrogen content of 400 mg. This quantity was added to the intake of amino acids in calculating the balance, but was not used in calculating the milligrams of amino acid nitrogen required for equilibrium.

The amino acid preparations were furnished us in bottles containing 2 kg of the powdered material. The accompanying nitrogen analyses were confirmed by us by macro-Kjeldahl analyses. In these, the digestion was performed in 500-ml Kjeldahl flasks with concentrated sulfuric acid, potassium sulfate and mercuric oxide. The ammonia produced by addition of sodium hydroxide and sodium sulfide was distilled into a boric acid solution and titrated with 0.01 N hydrochloric acid. An amount of amino acid preparation slightly in excess of that

[4] Kindly furnished by H. W. Kinney and Sons, Columbus, Indiana.

[5] Multicebrin-Lilly, kindly furnished by Eli Lilly and Company, Indianapolis, Indiana.

required for the day for the whole group of subjects was weighed out daily on a balance with an error of less than 0.1% and made up exactly into a 10% solution with distilled water in volumetric flasks. The weighing was done rapidly and the bottle containing the bulk of the amino acids was quickly restoppered to prevent uptake of moisture. Analyses at the end of the experiment showed no appreciable change in the nitrogen content of the amino acid powder. The exact quantity of the 10% solution was measured out for each subject with accurate pipettes, diluted with a cartose solution and divided into three equal portions. One portion was consumed at each of the three meals, at 8:00 A.M., 12:30 P.M., and 5:30 P.M. Additional carbohydrate in the form of a special candy or carbohydrate drink was allowed in the evening.

The caloric intake was from 50 to 60 cal. per kilogram of body weight from sources other than the amino acids. This large caloric intake had previously been found necessary by Rose ('49) to produce nitrogen balance when the only nitrogen source was amino acids. In spite of this high caloric intake, only one subject gained weight. Five of the remaining subjects lost a little weight, the net loss for all 7 subjects being 0.6 kg. The most significant loss of weight (1.8 kg) occurred in subject 2, who, as will be seen later, showed the most irregular pattern of nitrogen balance.

The subjects were intelligent and cooperative. They ate all their meals together under the supervision of a dietitian. Urine specimens were collected in stoppered bottles containing toluene and were brought to the laboratory by the subject each morning, including Sunday. Stools were collected in two-quart triple enamel jars previously marked for 1,000 ml. These stools were analyzed daily after preliminary homogenization directly in the jars with water and concentrated sulfuric acid and dilution to 1,000 ml. An aliquot of the homogenized material was taken for micro-Kjeldahl analysis according to the method of Hoffman ('41). Urine samples were similarly analyzed by the micro-Kjeldahl method but these analyses were frequently checked by the macro-Kjel-

dahl technic described above. The balances were calculated for each period from the average daily nitrogen excretion in the urine and stools rather than from the individual daily excretions, for the overlap of excretions, especially of the stools, produced a slight but annoying fluctuation of the daily balance.

The urine specimens were also analyzed for creatinine as a check on the completeness of the collections. Also, blood specimens at the beginning and the end of the study were analyzed for non-protein nitrogen, serum albumin and globulin, and serum cholesterol. Since these values were all found to be within normal limits and to show no significant change, they are not included in this report.

RESULTS

The average balance for each of the successive periods for each subject is shown in figures 1 to 7 inclusive. The nitrogen requirement for equilibrium (which hereafter will be designated NE) could be determined within narrow limits in most subjects. In subject 1 negative balance first occurred with 44 mg nitrogen per kilogram of the unsupplemented product; positive balance recurred when the intake was raised to 49. Therefore NE was taken as the mean of these values, 46.5 mg per kilogram per day. Subject 2 experienced negative balance first on 74 mg per kilogram per day, but he came into positive balance again on 79 and 76 mg. At the time of negative balance he was quite fatigued from hard work and late hours. When the nitrogen intake was dropped to 54 mg per kilogram he went into negative balance, and just failed to reach positive balance on 70 mg, which value was tentatively chosen as NE. Subject 3 was in negative balance with 50 mg per kilogram but in positive balance with 54. Therefore 52 mg was chosen as the NE value. Similarly, NE for subjects 4, 5, 6 and 7 was taken as 52, 52, 58.5 and 52 mg nitrogen per kilogram per day, respectively.

In the balance studies with the supplemented amino acid product, all subjects were started on 30 mg per kilogram per day but all were in negative balance on this regimen. On 35 mg only subject 3 came into approximate equilibrium, and that value was chosen as NE for him. Subjects 4 and 5 were in almost exact equilibrium at 40 mg per kilogram per day.

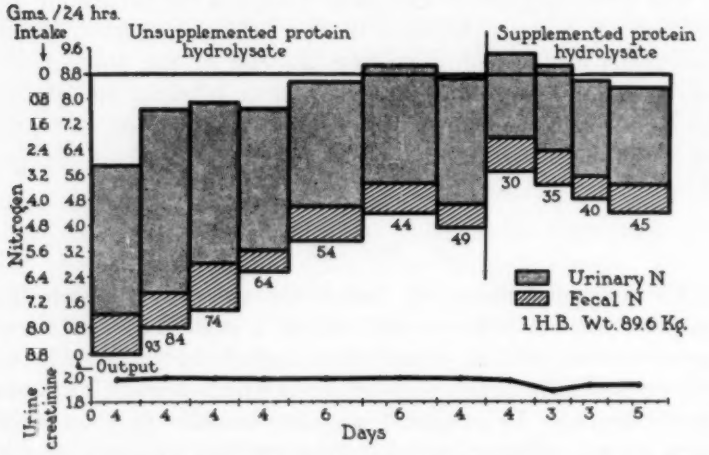

Fig. 1 Nitrogen balance study on subject 1 (H.B.). The average daily nitrogen intake (including that of the basal diet) for each period is expressed as a column down from the zero line. The output is shown as a column up from the bottom of the intake column, the stool nitrogen being represented by the cross-hatched portion. If the output is greater than the intake, its top will appear above the zero line to a height equal to the negative balance. If the balance is positive, the top of the output column will be below the zero line by a distance equal to the positive balance. The number under each column is the nitrogen intake supplied by the amino acid mixture, in milligrams per kilogram per day.

For these subjects, 40 mg was chosen as NE. In the other subjects, NE was taken as the mean of the nearest values producing negative and positive balances respectively. It was unfortunate that the experiment could not be prolonged at this stage to confirm the NE values by getting the subjects in and out of negative balance. But the results were

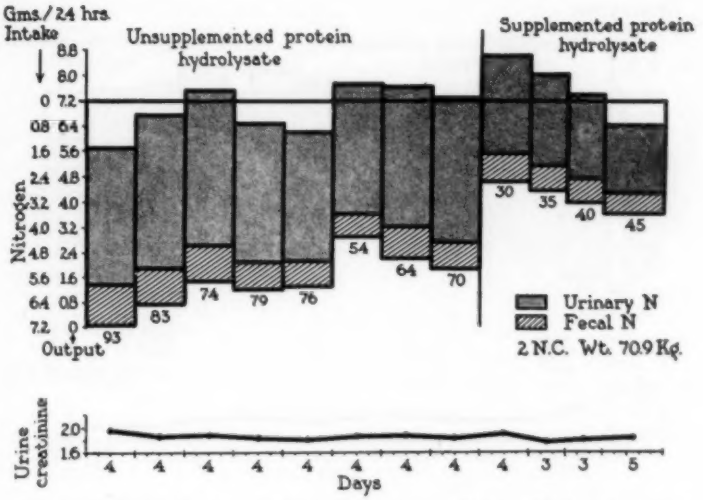

Fig. 2 Nitrogen balance study on subject 2 (N.C.). Explanation as for figure 1.

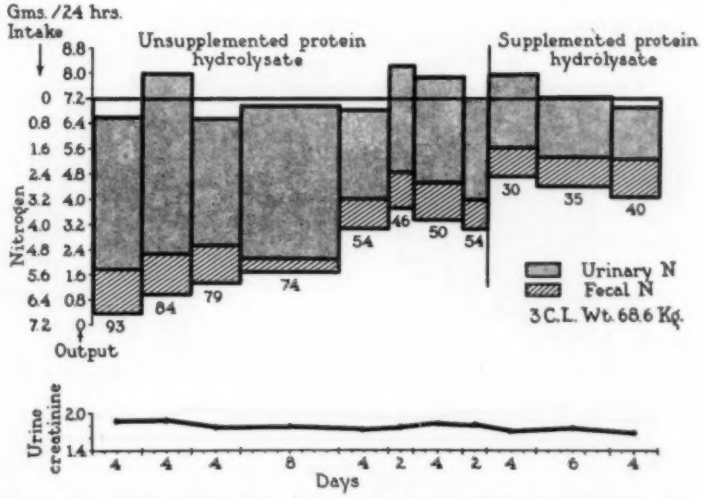

Fig. 3 Nitrogen balance study on subject 3 (C.L.). Explanation as for figure 1.

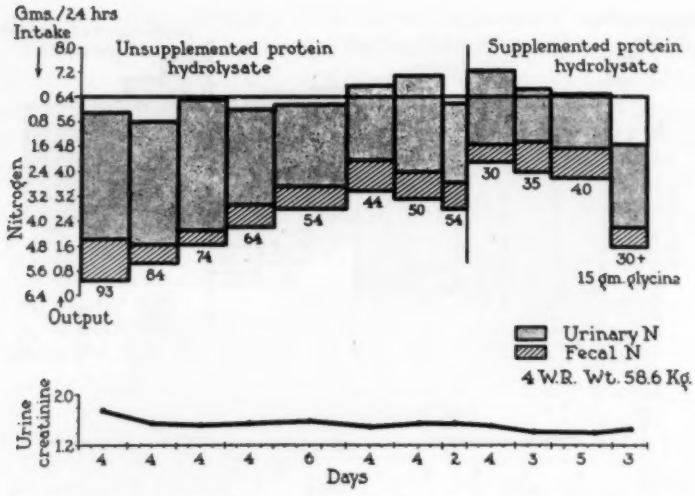

Fig. 4 Nitrogen balance study on subject 4 (W.R.). Explanation as for figure 1.

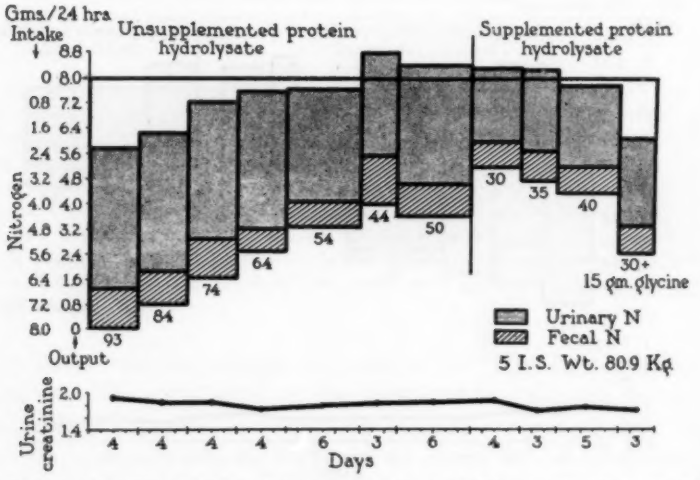

Fig. 5 Nitrogen balance study on subject 5 (I.S.). Explanation as for figure 1.

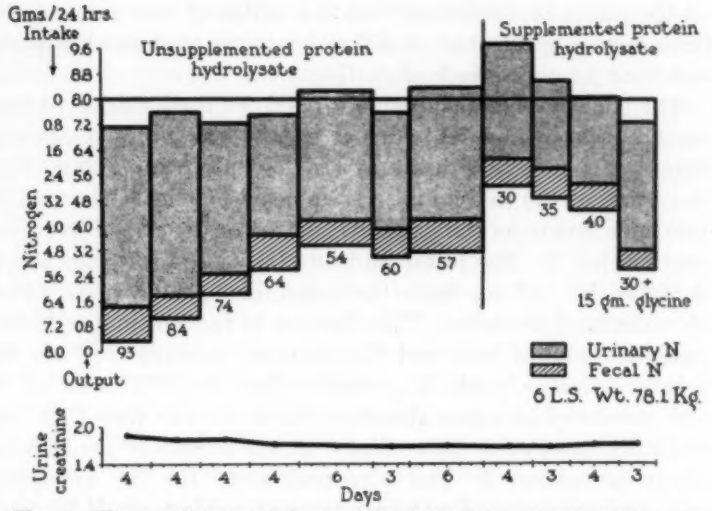

Fig. 6 Nitrogen balance study on subject 6 (L.S.). Explanation as for figure 1.

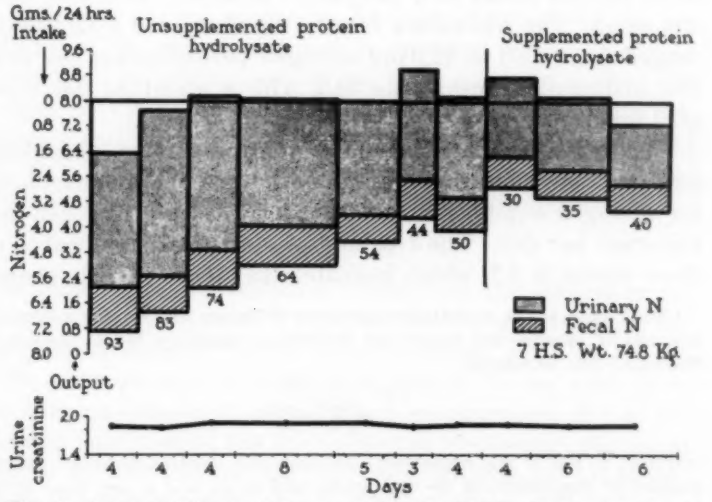

Fig. 7 Nitrogen balance study on subject 7 (H.S.). Explanation as for figure 1.

on the whole so consistent that it is unlikely that they would have been appreciably modified by more prolonged experiments or longer periods of testing.

The uncorrected mean value for NE for the 7 subjects when on the unsupplemented hydrolysate was 54.7 mg nitrogen per kilogram per day, with a standard deviation of 7.0 mg. The deviation of the individual NE values from the mean was of the same order as the standard deviation in all subjects except subject 2. The latter had an NE value of 70 mg, which deviated by 15.3 mg from the mean, or by more than twice the standard deviation. This finding in association with this subject's weight loss and the unusual character of his nitrogen balances made it probable that the NE value of 70 was produced by some abnormal state. It was therefore statistically more accurate to throw out the results of the balance study of subject 2. The corrected mean for the unsupplemented hydrolysate for the remaining 6 subjects was 52.2, with a standard deviation of 3.5. It was significant that the modal value of 52.0 found in 4 subjects was almost identical with this mean. The NE values for the supplemented hydrolysate ranged from 35.0 to 42.0 mg nitrogen per kilogram per day. The arithmetical mean was 38.5, with a standard deviation of 2.1 mg.

Thus it appears that the addition of tryptophan, phenylalanine and methionine significantly lowers the requirements for nitrogen equilibrium, from 52.2 to 38.5 mg nitrogen per kilogram per day. The t value [6] of statistical significance of these means is 5.1, which indicates that there is little chance

[6] The t value of the statistically significant difference between the average of two sets of experimental values was determined essentially by the method of Snedecor ('46), as follows:

$$t = \frac{a_1 - a_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

where a_1, σ_1, and n_1 are, respectively, the mean, the standard deviation, and the number of experiments in the first group, and a_2, σ_2, and n_2 are those of the second group. Values for t greater than 2.5 are regarded as indicating a significant difference in the means.

of this difference being fortuitous. Accordingly, supplementation of this casein hydrolysate with the three limiting essential amino acids converts it into a product of much higher biological value. Even without the supplement, however, the original lyophilized hydrolysate is seen to be an excellent protein product. The daily requirement for a 70-kg person averages 29.1 gm.

An additional balance experiment was carried out in three of the subjects in the last three available days. Subjects 4, 5, and 6 were given 30 mg per kilogram per day of the supplemented hydrolysate, a quantity which had previously been insufficient to produce nitrogen equilibrium. In addition to the supplemented hydrolysate, 15 gm of glycine, C.P., were given daily to raise the nitrogen content of the diet by 2.80 gm. It was unfortunate that the experiment could not be prolonged beyond three days, for in these three days each of the subjects showed large positive balances. The possible significance of these findings will be discussed below.

The daily creatinine excretions were on the whole constant in each subject, but tended to be slightly higher at the beginning of the experiment and to diminish gradually as the experiment progressed to the negative balances during the feeding of the supplemented product. What metabolic significance this slight decline had could not be determined. At any rate, there were no sharp fluctuations in the creatinine excretion that might indicate incomplete collections.

DISCUSSION

In table 1 Rose's tentative pattern of the minimum requirements of the essential amino acids is compared with the quantitative distribution of essential amino acids in 100 gm of the unsupplemented casein hydrolysate. There were from 9 to 12.5 equivalents of the minimum requirements of 5 of the essential amino acids, but the amount of tryptophan was equal only to 3.3 equivalents, that of phenylalanine to 5.0 equivalents and that of methionine to 3.0 equivalents. The limiting factors in the biological efficiency of the hydrolysate were

obviously these latter three amino acids. By trebling L-tryptophan and methionine and doubling L-phenylalanine, the amount of the resulting mixture required for equilibrium should be from one-half to one-third of what is required without the supplement (provided that the total nitrogen content of the intake is adequate for the body needs of nitrogen

TABLE 1

Relation of the quantitative pattern of essential amino acids in the unsupplemented hydrolysate to that of the minimum requirements of essential amino acids

	TENTATIVE MINIMUM REQUIREMENT	QUANTITY IN 100 GM HYDROLYSATE [2]	EQUIVALENTS
	gm	*gm*	
L-Tryptophan	0.15 [1]	0.5	3.3
L-Phenylalanine	1.10	5.5	5.0
L-Lysine	0.80	8.5	10.6
L-Threonine	0.40 [1]	5.0	12.5
L-Valine	0.80	7.3	9.1
L-Methionine	1.00 [1]	3.0	3.0
L-Isoleucine	0.70	7.4	10.6
L-Leucine	1.10	10.6	9.6

[1] These data were kindly supplied by Rose before publication. In his published paper ('49), Rose has changed the "tentative minimal requirement" for tryptophan to 0.25 (see text footnote 7), threonine to 0.50, and methionine to 1.10.

[2] These analyses were provided by the manufacturer.

and that other amino acids, such as cystine or tyrosine, do not reach limitingly low levels).

As far as the balance studies on the unsupplemented hydrolysate are concerned, the findings offer complete corroboration of Rose's calculations. The average intake of 52.0 mg nitrogen per kilogram per day of the unsupplemented hydrolysate is equivalent to 418 mg of the hydrolysate. For a 70-kg

person the quantity required is 29.2 gm. This quantity contains 0.15 gm of L-tryptophan and 0.88 gm of methionine, values very similar to the minimum requirements of these two amino acids as originally found by Rose [7] (see table 2). Nitrogen balance probably could not be maintained on lower quantities of the hydrolysate, even with the total nitrogen and the other essential amino acids in excess.

On the other hand, when the subjects were placed on the diet of hydrolysate supplemented with the three limiting amino acids in the proportion shown in table 2, they went into negative balance long before the limiting value of these three or any of the other 5 essential amino acids had been reached. In fact, as can be seen from table 2, at the average value for nitrogen equilibrium the quantity of each essential amino acid present in the diet for a 70-kg individual was about twice that of the minimum requirement found by Rose. The average nitrogen requirement of 38.5 mg per kilogram was, it is true, significantly lower than that for the unsupplemented product — about 26% lower; but if the limiting factors were only the quantities of essential amino acids, nitrogen equilibrium should have been maintained with about 20 mg per kilogram per day.

These findings have led us to believe that in such an ideal amino acid combination the limiting factor is no longer the quantity of essential amino acids but the total amount of nitrogen which the body can utilize for the building of missing "non-essential" amino acids and other nitrogenous products. That this may be the case is indicated by the short experiment when 2.8 gm of nitrogen in the form of glycine were added to a diet of 30 mg of nitrogen per kilogram per

[7] In Rose's recent publication ('49) he suggests that the minimum requirement for L-tryptophan may have to be raised to 0.25 gm daily because one subject out of 12 studied needed that quantity. The remaining 11 needed less than 0.2 gm. Our present findings are in corroboration of Rose's earlier value of 0.15 gm, if the D-tryptophan present in our mixtures had no nutritive value.

TABLE 2

Relation of quantities of essential amino acids in daily requirements of unsupplemented and supplemented hydrolysate to those found by Rose

Unsupplemented amino acids

Average requirement for nitrogen equilibrium:
52.2 mg per kilogram per day
= 417.6 mg unsupplemented amino acids
(N × 100/12.5; Elamine, I.C.-lyophilized)
= 29.23 gm amino acids per day per 70-kg individual

Supplemented amino acids

Average requirement for nitrogen equilibrium:
38.5 mg N per kilogram per day
= 316.4 mg supplemented amino acids
(N × 100/12.17)
= 22.15 gm amino acids per day per 70-kg individual

	QUANTITY OF AMINO ACIDS IN 29.23 GM UNSUPPLEMENTED	QUANTITY OF AMINO ACIDS REQUIRED DAILY AS FOUND BY ROSE [1]	QUANTITY OF ESSENTIAL AMINO ACIDS IN 22.15 GM SUPPLEMENTED PRODUCT [2]
	gm	gm	gm
L-Tryptophan	0.15	0.15	0.10 + 0.20 = 0.30
L-Phenylalanine	1.61	1.10	1.06 + 1.19 = 2.25
L-Lysine	2.48	0.80	1.64
L-Threonine	1.46	0.40	0.96
L-Valine	2.13	0.80	1.41
L-Methionine	0.88	1.00	0.58 + 1.36 = 1.94
L-Isoleucine	2.05	0.70	1.43
L-Leucine	3.10	1.10	2.04

[1] Personal communication.

[2] Four thousand grams of supplemented lyophilized casein hydrolysate contain the following:

casein hydrolysate + 1% DL-tryptophan	3,480.0 gm
DL-tryptophan	70.8 gm
L-phenylalanine	214.0 gm
DL-methionine	246.0 gm

Also 22.15 gm of the supplemented product contain 19.27 gm unsupplemented hydrolysate
+ 0.39 gm DL-tryptophan or 0.20 gm L-tryptophan
+ 1.19 gm L-phenylalanine
+ 1.36 gm DL-methionine

day of the supplemented hydrolysate. Whereas in the earlier period 30 mg had been insufficient to produce positive balance, that quantity with the addition of glycine produced strongly positive balance in all three subjects.

The experiment of three days was entirely too brief to be conclusive. It should be repeated and enlarged. Indeed, it might be desirable to repeat the balance studies with the modification of having 15 gm of glycine as a constant factor throughout all the periods of feeding varying quantities of unsupplemented and supplemented hydrolysate. Such an experiment might determine whether the supplemented hydrolysate is really three times as effective as the unsupplemented one, as is suggested by Rose's findings, or whether other limiting factors are involved. The special value of additional nitrogen in the form of non-essential amino acids has recently been shown for rat growth by Russell and Taylor ('48), but it has heretofore not been demonstrated quantitatively for human subjects.

If the glycine experiment can be corroborated, it would appear that the supplemented hydrolysate would be just as good a protein product if it were diluted with some glycine. Indeed, if no other limiting factors in the mixture are found and if Rose's pattern of minimum requirements of the essential amino acids still holds when all are at a minimum, the dilution could be made with an equal quantity of glycine. However, this inference remains to be proved.

SUMMARY

Nitrogen balance experiments were performed in 7 normal male human subjects to determine the nitrogen requirements for equilibrium when the only significant source of nitrogen was a lyophilized modified casein hydrolysate [8] given orally. Then in the same subjects the nitrogen requirement for equilib-

[8] See footnote 3, page 124.

rium was determined for the same hydrolysate fortified with DL-tryptophan, L-phenylalanine, and DL-methionine in quantities that made the quantitative pattern of essential amino acids in the supplemented product equivalent to that found by Rose in his estimation of the minimum requirements of the individual amino acids in human subjects.

The corrected mean nitrogen requirement for equilibrium with the unsupplemented hydrolysate was 52.2 mg per kilogram per day, with a standard deviation of 3.5 mg. For the supplemented product the mean nitrogen requirement for equilibrium was 38.5 mg per kilogram per day, with a standard deviation of 2.1 mg, a reduction of 26%. This reduction was found to be statistically significant.

Nitrogen equilibrium was reached with the unsupplemented product when L-tryptophan and methionine were present in the diet in quantities of the order of the minimum amounts found to be required by Rose. Therefore, these amino acids were the limiting factors determining the amount of nitrogen in the product required for equilibrium. On the other hand, nitrogen equilibrium was reached with the supplemented products when all the essential amino acids were still present in twice as great a quantity as Rose had found necessary to meet the minimum requirements. Therefore the limiting factor in the supplemented product appeared to be the total quantity of nitrogen present. This conclusion was corroborated in a short experiment in which the addition of 15 gm of glycine changed from negative to positive the balance produced by a diet of 30 mg per kilogram per day of the supplemented product.

ACKNOWLEDGMENTS

The authors wish to acknowledge gratefully their indebtedness to Mrs. Irene Maxwell who, as dietitian for the project, prepared all diets and recipes, and to Mrs. Lorraine Schmelzle Richter and Miss Frances Gillman for the chemical analyses.

APPENDIX

Basic diet:

Breakfast	*Lunch*	*Supper*
Orange beverage	Soup	Soup
Low nitrogen fruit	Cornstarch pudding	Low nitrogen fruit
Nitrogen-free muffins	Nitrogen-free muffins	Nitrogen-free muffins
Jelly	High carbohydrate beverage	Candy
Black coffee	Candy	Beverage, tea or coffee
Sugar	Vitamin capsule	Sugar
	Between meals: Candy, as desired; High carbohydrate beverage, 1 quart per day	
	Bedtime: 1 Orange; Muffins; Candy; Finish high carbohydrate beverage	

Recipes:

Orange beverage

100 ml orange base, high carbohydrate beverage
100 ml fresh orange juice, strained

Nitrogen-free muffins
(For 6 muffins)

2 tablepsoons sugar
4 tablespoons cornstarch
4½ tablespoons tapioca flour
2 teaspoons baking powder
½ tablespoon cellu flour, add
1 tablespoon oil and resift

Then add 1 teaspoonful of flavoring, cinnamon or vanilla, a few drops of eggshade (added to water for even color) and water, 125 ml. Mix lightly and spoon out into a muffin pan. Bake in a 350–450°F. oven for 15 minutes or longer.

Note: Add 1 package of physiological salt mixture per person per day to dry ingredients.

Cornstarch pudding
(Single serving)

2 tablespoons cornstarch
2 tablespoons granulated sugar
150 ml water

Flavoring and color: strawberry, orange, lemon, or vanilla, plus eggshade, or juice from canned apricots to replace water in recipe.

Carrot soup
(Single serving)

200 ml water
30 gm carrots, strained or sliced

1 tablespoon oil plus salt, onion flakes, and pepper.

Tomato soup
(Single serving)

100 ml water
100 ml tomatoes, juice or canned (never puree)

1 tablespoon oil plus salt, onion flakes, and pepper.

Candy

2 cups sugar
½ cup cold water
¼ teaspoon cream of tartar

Stir over slow heat until sugar is dissolved. Then cook quickly without stirring to soft ball stage. Remove from fire, let stand for two minutes. Add flavoring and color. Beat until creamy. Drop on wax paper. Flavorings used: peppermint, clove, lemon, maple, orange, and cherry.

High carbohydrate beverage
(One quart)

250 ml cartose
1 tablespoon beverage powder
1 tablespoon lemon extract or juice

Dissolve in small amount of hot water, then make up to volume with cold water.

Vitamins

Multicebrin, Lilly, Gelseals no. 100

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

XVIII. THE EFFECT OF MULTI-VITAMIN SUPPLEMENTS ON THE SECRETION OF B VITAMINS IN HUMAN MILK [1]

J. P. PRATT AND B. M. HAMIL

Departments of Obstetrics, Gynecology and Pediatrics, Henry Ford Hospital, Detroit, Michigan

ELSIE Z. MOYER, MILDRED KAUCHER, CHARLOTTE RODERUCK,[2] MARGARET N. CORYELL, SOL MILLER, HAROLD H. WILLIAMS,[3] AND ICIE G. MACY

Research Laboratory of the Children's Fund of Michigan, Detroit

ONE FIGURE

(Received for publication December 18, 1950)

It is known that certain of the B vitamins participate, directly or indirectly, in the enzyme system of the body and thereby play significant roles in the intermediary metabolism of carbohydrate, certain essential amino acids, and fatty acids. Enlarged knowledge of the role of these vitamins in the physiology of milk secretion is especially pertinent to the maintenance of the nutritional status of the nursing mother and her infant. The present investigation was undertaken to study the levels of thiamine, riboflavin, niacin, pantothenic acid, and biotin in the milk and urine of nursing mothers: (1) while they were ingesting the same foods in

[1] The investigation represented in part by this paper was partially supported by a grant from the Nutrition Foundation, Inc., and was made possible through the cooperation of the mothers and their families and of Velma Davies, R.N., who supervised the manual expression of the 24-hour collections of breast milk.

[2] Now Assistant Professor in the Foods and Nutrition Department, Division of Home Economics, Iowa State College, Ames.

[3] Now Professor of Biochemistry, Cornell University, Ithaca, N. Y.

diets adjusted to meet physiologic needs and satisfy appetite; and (2) the corresponding rates of elimination of these vitamins in milk and urine after their self-chosen diets had been supplemented for 6 to 14 days with approximately 10 times the recommended daily allowances of thiamine, riboflavin, niacin, vitamin A, and vitamin C.

EXPERIMENTAL

Ten mothers 21 to 35 years old participated in the study during one to 4 periods of observation after mature milk production was established. Observation periods were between 45 and 316 days post partum and all collection procedures were carried out in the homes while the women continued their usual activities. All of the subjects were healthy multiparas who were successfully nursing their infants. Their body weights ranged from 46.3 to 73.9 kg; their heights from 152 to 173 cm; and their surface areas from 1.4 to 1.9 m^2. Results of determinations of pulse and respiration rates, body temperature, blood pressure, respiratory quotient, and basal metabolic rate were all within the ranges accepted as normal.

During the 18 control periods of 5 consecutive days for the 10 women, energy, thiamine, riboflavin, niacin, pantothenic acid, and biotin were determined in aliquot samples of the food eaten and in consecutive 24-hour collections of breast milk and, except energy, in the total daily excretions of urine. To assure each woman's ingesting the same distribution of foods during the 5 days, all food was provided by the laboratory and its use supervised by a member of the laboratory staff. The diets were designed to meet physiologic requirements as they are known but total food intake was determined by appetite.

Immediately following her control period, each mother received daily for 6 to 14 days a supplement consisting of 750 mg ascorbic acid, 50,000 I.U. vitamin A, 12 mg thiamine chloride, 15 mg riboflavin, and 120 mg niacin — amounts less than 10 times the allowances recommended by the Food and

Nutrition Board ('48) but similar to the quantities often taken by women. The ascorbic acid supplement was given in three equal portions, one with each meal, to permit fuller utilization. The other vitamin supplements were given with the morning meal.

During the periods of supplementation the diets were uncontrolled but the women were encouraged to avoid excessive intakes of candy and other sweets and to take only the vitamin supplement provided. Continuous records of the food eaten by the women indicated that their diets were nutritionally good but family patterns of food purchasing and preparation were shown by greater variability in the food intakes of different women and of the same woman from day to day than existed during the control periods. During the final day of supplementation, 24-hour collections of breast milk and urine were obtained for analyses. Representative samples of milk were assured by manual expression of all milk from both breasts at 4-hour intervals throughout the day.

Preceding papers have reported the procedures of collection and the analytical methods used (Macy, Williams, Pratt and Hamil, '45; Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Davies, '45). Thiamine was determined by the thiochrome method described by Hennessy ('42), with slight modifications (Roderuck, Williams and Macy, '45); riboflavin by the Conner and Straub method ('41) as described previously (Roderuck, Coryell, Williams and Macy, '45); and niacin, pantothenic acid and biotin by microbiological procedures, using *L. arabinosis* as the assay organism (Coryell, Harris, Miller, Williams and Macy, '45).

An earlier paper (Pratt, Munks and Macy, '47) showed that the major portion of the ascorbic acid dietary supplement was accounted for by increases in urinary excretion. Another communication (Lesher, Robinson, Brody, Williams and Macy, '48) presented data showing that additions of at least 50,000 I.U. of vitamin A per day are necessary in the diets of nursing women to provide any marked increase

in the vitamin A content of the breast milk. Whether increasing concentrations of vitamins in the milk of healthy nursing mothers consuming adequate mixed diets provide any additional benefits for their infants will be discussed in forthcoming papers.

RESULTS AND DISCUSSION

Table 1 presents the mean volumes of milk and urine per 24 hours for each subject during the 5-day control periods; the average daily amounts of thiamine, riboflavin, niacin, pantothenic acid, and biotin in the food intakes, in the milk secreted and urine excreted during the control period; and the volumes of milk and urine collected during the last day of administration of the supplement and the amounts of the vitamins in the milk secreted and urine excreted. The data for thiamine, riboflavin, and niacin are plotted in figure 1. The concentrations of the vitamins in the milk and urine are given in table 2.

The volumes of milk produced by the women portray their individual physiologic patterns, varying from 268 to 1,020 ml per day during the control periods and from 99 to 1,144 ml for the final 24-hour intervals of supplementation. During 15 of the 18 studies, 24-hour volumes increased during the 5 days by an average of 127 ml. The average decrease in three instances was 34 ml. However, of the 18 mean volumes for control periods, 9 were larger and 9 were smaller than the respective 24-hour volumes after 6 to 14 days of supplementation, indicating that neither the stimulation (Davies and Pratt, '46) of manual expression during the control period nor the administration of vitamin supplements exerted a major influence upon the volume of milk produced.

The average daily energy intakes of the women during the control periods ranged from 2,547 to 3,559 cal., with a range of 2,435 to 3,410 calories-not-fat. Considering the body weights of the women (table 1), the energy intakes conformed satisfactorily with the 3,000 cal. per day recommended by the Food and Nutrition Board ('48) for lactating women

weighing 56 kg. Analysis of samples representing the actual food intakes of the women during each of the 5 days showed that the mean amounts of thiamine, riboflavin, and niacin ingested daily were 87, 107, and 127%, respectively, of the amounts recommended for lactating women. By calculation from standard tables of food values, higher intakes were indicated (Kaucher, Moyer, Williams and Macy, '46). Per 1,000 cal., intakes of thiamine ranged from 0.38 to 0.53 mg; of riboflavin from 0.94 to 1.23 mg; and of niacin from 5.17 to 7.82 mg. Per 1,000 calories-not-fat, the range values were, respectively, 0.42 to 0.55 mg, 0.98 to 1.28 mg, and 5.39 to 8.17 mg.

No attempt was made to evaluate dietary samples which would represent the food intake from which were derived the vitamins in the milk and urine collected during the 24 hours after supplementation. In addition to the sampling and calculation problems, the question of a possible effect of the supplement upon appetite required consideration. However, it has been practical and justifiable, because the intakes of thiamine, riboflavin, and niacin from food were small in relation to the amounts included in the supplement, to assume that intakes for the day after supplementation were equivalent to the mean intakes for the 5-day control period. This preliminary study indicates the rapidity of individual physiologic adjustments to augmented intakes of vitamins; it was not designed to demonstrate the individual's maximum response, characteristic physiologic stabilization, or the requirement of the nursing mother, although the results provide some significant implications regarding these aspects of lactation.

The mean concentrations of thiamine, riboflavin, niacin, pantothenic acid, and biotin in the milk secreted by the women were equal to, or greater than, the average concentrations given for mature human milk in two recent, comprehensive reviews of the literature pertaining to the composition of milk (Kon and Mawson, '50; Macy, Kelly and Sloan, '50). The mean daily intakes of thiamine during the control pe-

TABLE 1

B vitamins in 24-hour collections of milk and urine from nursing mothers before and after ingestion of multi-vitamin dietary supplements

SUBJECT	INTERVAL, POST PARTUM	HEIGHT	WEIGHT	VOLUME				THIAMINE					RIBOFLAVIN				
				Control		After supplement		Control			After supplement		Control			After supplement	
				Milk	Urine	Milk	Urine	Intake	Milk	Urine	Milk	Urine	Intake	Milk	Urine	Milk	Urine
	days	*cm*	*kg*	*ml*	*ml*	*ml*	*ml*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
M.B.	72–76	160	51.2	718	691	1002	802	1.06	0.11	0.03	0.29	2.78	2.67	0.26	0.69	2.08	11.01
V.G.	78–82	157	59.9	848	1281	1004	950	1.36	0.13	0.32	0.23	4.40	3.17	0.41	1.44	2.13	11.06
	161–165	157	59.0	901	993	870	...	1.18	0.11	0.33	0.16	...	3.24	0.38	1.17	1.89	....
	239–243	157	58.0	681	810	721	922	1.18	0.09	0.32	0.15	4.20	3.39	0.29	1.21	1.62	10.55
	302–306	157	59.0	394	1080	320	956	1.38	0.06	0.37	0.08	5.34	3.12	0.18	1.28	0.72	11.11
V.K.	95–99	162	63.0	647	2804	694	1830	1.07	0.10	0.38	0.14	3.43	3.03	0.24	1.82	1.43	14.60
	144–148	162	57.6	325	1447	326	852	1.20	0.04	0.32	0.05	2.72	3.08	0.11	1.22	0.56	11.06
V.L.	68–72	159	57.2	789	2849	778	1752	1.50	0.13	0.24	0.22	4.68	3.47	0.25	1.39	1.24	10.59
	152–156	159	54.9	680	1756	520	1534	1.61	0.11	0.32	0.14	4.55	3.52	0.22	1.26	0.88	12.18
J.M.	75–79	155	55.3	708	2265	604	3371	1.38	0.10	0.28	0.15	3.70	3.41	0.33	1.50	1.54	12.02
	173–177	155	56.2	268	2003	99	1813	1.59	0.03	0.30	0.01	2.99	3.33	0.11	1.12	0.07	10.68
D.M.	45–49	173	60.3	401	1741	289	2245	1.36	0.06	0.22	0.07	3.33	...	...	...	...	....
B.S.	85–89	161	59.0	1020	1347	1144	1155	1.11	0.16	0.17	0.28	3.71	3.08	0.46	1.33	2.58	12.23
	204–208	161	58.0	913	1356	973	1612	1.32	0.16	0.33	0.25	4.60	3.29	0.45	1.80	1.73	11.80
	259–263	161	59.4	676	1219	649	1097	1.26	0.12	0.34	0.19	0.53	3.22	0.33	1.75	1.44	12.71
M.S.	58–62	152	46.3	947	939	1037	610	1.17	0.15	0.16	0.25	4.11	3.36	0.36	1.37	1.82	11.19
G.S.	80–84	170	64.4	899	1078	992	1140	1.41	0.13	0.26	0.24	5.38	2.93	0.41	0.95	1.93	....
V.S.	70–74	173	73.9	304	1852	128	1376	1.40	0.04	0.80	0.02	1.99	3.34	0.12	2.05	0.08	3.87

TABLE 1 *(continued)*

vitamins in 24-hour collections of milk and urine from nursing mothers before and after ingestion of multivitamin dietary supplements

NIACIN					PANTOTHENIC ACID					BIOTIN				
Control			After supplement		Control			After supplement		Control			After supplement	
Niacin		F_2	Niacin	F_2										
Intake	Milk	Urine	Milk	Urine	Intake	Milk	Urine	Milk	Urine	Intake	Milk	Urine	Milk	Urine
mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mmg*	*mmg*	*mmg*	*mmg*	*mmg*
14.78	1.34	..	5.77	...	6.89	1.17	2.20	1.90	1.97	51.82	0.90	8.21	8.08	15.43
23.36	2.02	..	4.19	...	7.76	2.24	4.70	2.47	3.88	88.13	9.57	43.08	10.42	29.44
19.43	1.83	0.9	3.19	...	8.62	2.16	4.72	1.88	...	73.32	5.13	36.75	2.00	....
17.00	1.16	4.4	2.36	26.8	7.99	1.36	3.80	1.38	2.89	96.29	4.33	39.69	3.26	18.87
18.66	0.64	4.6	1.03	28.6	7.58	1.05	4.74	0.71	4.17	90.85	4.03	39.84	1.09	13.98
14.60	1.30	0.8	2.52	...	8.65	1.85	5.10	2.26	6.41	77.57	8.40	52.40	13.12	56.32
14.72	0.56	2.4	0.96	19.6	8.16	0.98	4.50	0.83	3.05	65.05	1.67	23.68	3.48	29.32
21.65	1.77	4.0	3.77	51.8	9.25	2.28	3.64	2.30	3.05	71.32	5.48	28.85	6.19	24.61
21.22	1.17	4.0	2.24	30.2	9.40	1.96	3.91	1.39	3.41	98.48	6.35	39.22	3.91	21.01
20.15	1.57	4.7	3.84	72.6	9.42	2.13	5.12	2.05	5.38	82.93	5.12	36.71	5.06	25.00
20.54	0.52	5.5	0.18	47.2	8.12	0.78	4.75	0.19	3.71	116.85	1.86	38.20	1.00	14.62
....	...	..	...	...	...	...	...	...	...	....	...	.....	...	....
19.06	1.71	..	5.51	...	6.78	2.05	4.23	2.38	2.99	68.21	5.63	25.13	5.26	36.32
18.93	2.00	..	4.95	...	7.08	2.09	5.18	2.00	3.77	79.49	10.82	50.91	4.70	27.10
20.56	1.40	..	2.08	...	8.17	1.85	5.17	2.06	4.73	74.57	8.26	52.13	7.33	35.90
18.35	1.31	..	4.26	...	9.08	2.04	4.18	1.81	3.02	82.93	9.86	45.91	3.59	19.28
20.76	1.65	..	2.28	...	6.70	2.30	3.87	2.56	3.11	73.18	6.26	28.78	2.48	17.27
20.14	0.86	8.8	0.37	11.3	6.93	0.88	6.17	0.41	6.24	82.20	3.04	54.10	3.40	39.94

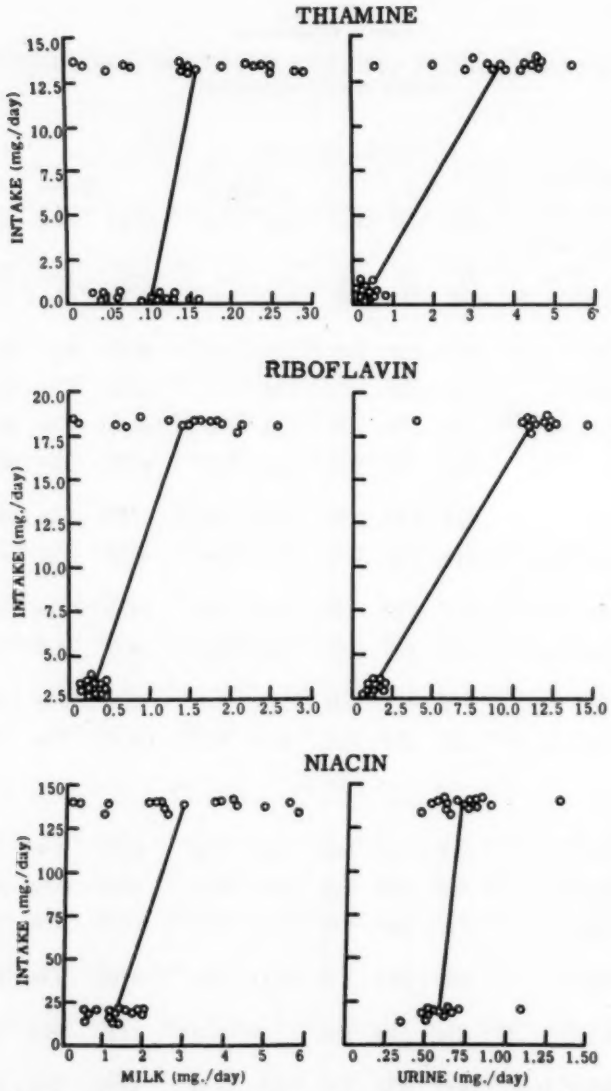

Fig. 1 Mean daily amounts of thiamine, riboflavin, and niacin in food intakes, milk secreted, and urine excreted during control periods versus amounts in intakes, milk, and urine after administration of synthetic supplements.

TABLE 2

The concentration of B vitamins in the milk and in the urine of nursing mothers before and after ingestion of multi-vitamin dietary supplements (Values per 100 ml)

SUBJECT	INTERVAL POST PARTUM	THIAMINE				RIBOFLAVIN				NIACIN				PANTOTHENIC ACID				BIOTIN			
		Control		After supplement		Control		After supplement		Control		After supplement		Control		After supplement		Control		After supplement	
										Niacin	F_2	Niacin	F_2								
		Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine	Milk	Urine
	days	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*	*mμg*
M.B.	72–76	15	4	29	347	36	100	208	1373	187	...	576	...	163	319	190	246	0.12	1.19	0.81	1.92
V.G.	78–82	15	25	23	464	49	112	212	1164	238	...	417	...	265	367	246	408	1.13	3.36	1.04	3.10
	161–165	12	33	18	...	42	118	217	....	203	0.09	367	...	240	476	217	...	0.57	3.70	0.23	...
	239–243	13	40	21	456	42	149	225	1144	171	0.54	327	2.90	199	469	191	313	0.64	4.90	0.45	2.05
	302–306	16	35	25	559	46	118	226	1162	162	0.43	321	3.00	267	439	223	436	1.02	3.69	0.34	1.46
V.K.	95–99	15	14	20	187	38	65	207	798	202	0.03	363	0.00	286	182	325	350	1.28	1.87	1.89	3.08
	144–148	12	22	16	320	33	84	174	1302	171	0.17	294	2.31	303	311	254	358	0.51	1.64	1.07	3.44
V.L.	68–72	16	8	28	267	32	49	159	604	225	0.14	485	2.96	289	128	296	174	0.69	1.01	0.80	1.40
	152–156	16	18	27	297	32	72	169	794	172	0.22	430	1.97	288	222	268	222	0.93	2.23	0.75	1.37
J.M.	75–79	14	13	25	110	46	66	254	357	221	0.21	635	2.15	300	226	340	160	0.72	1.62	0.84	0.74
	173–177	11	15	6	165	42	56	72	589	193	0.28	178	2.60	290	237	193	205	0.69	1.91	1.01	0.81
D.M.	45–49	16	12	24	148	..	..	..	...	...	...	...	...	...	...	...	...	...	...	...	...
B.S.	85–89	16	13	24	321	45	99	225	1059	168	...	482	...	201	314	208	259	0.55	1.86	0.46	3.14
	204–208	17	24	26	285	49	133	178	732	219	...	509	...	229	382	206	234	1.18	3.75	0.48	1.68
	259–263	17	28	29	48	49	143	223	1158	206	...	321	...	274	424	317	431	1.22	4.28	1.13	3.27
M.S.	58–62	16	18	24	674	38	146	176	1835	138	...	411	...	216	445	175	496	1.04	4.89	0.35	3.16
G.S.	80–84	15	24	25	472	46	103	195	....	183	...	230	...	256	359	258	273	0.70	2.67	0.25	1.51
V.S.	70–74	14	43	15	144	39	111	62	281	283	0.47	288	0.82	288	333	319	453	1.00	2.92	2.66	2.90

riods ranged from 1.06 to 1.61 mg (table 1), of which 2 to 15% (mean, 8%) was secreted in breast milk and 2 to 57% (mean, 23%) was excreted in urine. Individual physiologic differences are apparent among the data for different women and for the same woman at various stages of mature milk production. After 10 days of receiving supplements which included 12 mg thiamine chloride, superimposed upon a good diet, the 24-hour thiamine secretion in milk increased, on the average, 160% over the amounts found for the control period. The average thiamine concentration in milk increased 47% from 15 mμg/100 ml (range, 11 to 17 mμg) to 22 mμg/100 ml (range, 6 to 29 mμg).

In this test of vitamin additions to diets the indications are that the nursing mother responds rapidly to thiamine therapy, but it is unlikely that in such short intervals all of the women attained equilibrium, with thiamine concentrations stabilized at their full individual characteristic physiologic levels, even though their nutritional states were satisfactory with respect to thiamine as judged by fasting urinary thiamine excretions (Committee on Nutrition Surveys, '49). Of the total daily intake of thiamine from food and the supplement, an average of 70% was unaccounted for in the milk or urine, approximately the percentage unaccounted for during the control periods. After supplementation the mean daily secretion of free thiamine in milk was increased 125% and the concentration 133%.

Vitamin supplementation increased manyfold the daily amounts excreted in the widely varying volumes of urine produced. The mean amount of vitamins in urine increased 12 times with an average intake 10 times greater than that of the control period. The total output of thiamine in milk and urine increased from an average of 0.40 mg per day to 3.83 mg with intakes increased from a mean of 1.31 to 13.31 mg per day. Without data for the amounts in the feces the quantity of thiamine absorbed from the 9 mg, approximately, which were not accounted for in milk or in urine cannot be determined. Several decomposition products result

from the metabolism of thiamine, the principal ones being pyrimidine and thiazole. At the time of this study neither the presence nor the amount of these compounds and their derivatives were determined, which may explain, in part, the large quantities of thiamine unaccounted for. The excretion of these compounds, which represent a substantial portion of the intake, appears to be related linearly to thiamine intake but is highly characteristic of the individual (Mickelsen, Caster and Keys, '47).

Of the mean daily intakes of riboflavin during the control periods, ranging from 2.67 to 3.52 mg, an average of 9.0% (range, 3.3 to 14.9%) was secreted in breast milk and an average of 43% (range, 26 to 61%) was eliminated in urine. After consumption of 15 mg of riboflavin in the daily supplement, the mean concentration of riboflavin per 100 ml of breast milk increased about 5 times when intake was increased about 6 times. The per cent of riboflavin intake appearing in the milk, 7.7%, was only slightly less than before supplementation. It would appear that, at these levels of riboflavin intake, the daily output of riboflavin in milk is directly proportional to the intake.

The urinary excretion of riboflavin increased approximately 8 times following supplementation. When the intake was about 3 mg per day, 43% was accounted for in the urine; when the intake was about 18 mg per day, 61% was found in the urine. Similar to the effect of the synthetic supplement upon the output of free thiamine in milk, the daily secretion of free riboflavin in milk was increased 650% and the concentration 614%.

Marked fluctations in the riboflavin content of milk have been shown to occur during the day owing to the influence of the amounts in the individual meals of the mother (Kon and Mawson, '50). Twenty-four-hour collections obscure diurnal variations but do reflect the current consumption of riboflavin and alterations concurrent with progression in lactation (Roderuck, Coryell, Williams and Macy, '45, '46). The urinary excretion of vitamin calculated as excretion per hour

as a means of evaluating vitamin nutriture is perhaps less meaningful with respect to riboflavin than for thiamine. As judged by fasting urine tests, however, the nutrition of the subjects of this study was satisfactory with respect to riboflavin because all but 4 showed urinary excretions greater than 20 mμg of riboflavin per hour.

Intestinal synthesis of riboflavin does take place in women but it is difficult to determine how much of the product is absorbed or utilized (Davis, Oldham and Roberts, '46; Harris and Scoular, '49). Fecal excretion of riboflavin, however, does not appear to be markedly influenced by dietary levels unless the diet is an unusual one. It is unlikely that the elimination levels in milk and urine in the present study were influenced greatly by riboflavin originating from intestinal synthesis.

Studies on the utilization of niacin by nursing mothers and the breast milk content of the vitamin have been reported (Coryell, Harris, Miller, Williams and Macy, '45; Coryell, Roderuck, Harris, Miller, Rutledge, Williams and Macy, '47). Niacin is an integral part of the pyridine nucleotide coenzymes that function in the oxidative enzyme systems necessary to cell respiration in the organism. It may be synthesized, however, from tryptophan (Kallio and Berg, '49; Olcese, Pearson and Sparks, '49) and is important in protein metabolism. In the present investigation the average daily niacin intakes of the mothers during the control periods ranged from 14.6 to 23.4 mg, of which an average of 7% (range, 2 to 10%) was found in the milk and an average of 3% (range, 2 to 5%) was excreted in the urine.

Following consumption of the multi-vitamin supplement, which included 120 mg of niacin, the niacin content of the milk increased, on the average, 117% (range, 38 to 330%) and the average amount in the urine 22% (range, 6 to 96%). The mean concentration of niacin in milk was 196 mμg/100 ml (range, 138 to 283 mμg) during the control period. After supplementation the mean concentration was 390 mμg (range, 178 to 635 mμg), an increase of 99%. The average niacin concentrations in urine increased 43 to 58 mμg following in-

gestion of the vitamin supplements. Of the two mothers whose milk flow was ceasing, the concentration of niacin in the milk of one (J.M.) decreased but it did not change appreciably in the secretion of the other subject (V.S.). The major portion of the niacin secreted in milk was in the free form. The data suffice to demonstrate that the niacin concentration of breast milk can be altered in a relatively short time by addition of synthetic niacin to the diet.

In 8 study periods the urinary F_2 (N^1-methylnicotinamide chloride) values before and after supplementation ranged from 2.4 to 8.8 mg per day and 11.3 to 72.6 mg per day, respectively.

Since many of the vitamins may function synergistically, determinations were included of the secretion in milk and excretion in urine of pantothenic acid and biotin before and after supplementation of the diets, although neither nutriment was included in the vitamin supplement. No consistent physiologic effect was observed upon either the daily amounts or concentrations of pantothenic acid and biotin in milk. Similarly, the multi-vitamin supplement did not produce a definite effect upon the urinary excretion of these nutriments. These results indicate that during full lactation amounts of thiamine, riboflavin, niacin, vitamin A, and ascorbic acid somewhat less than 10 times the recommended allowances do not alter metabolic trends in the secretion of biotin and pantothenic acid in breast milk nor their excretion in the urine during observation periods of several days. This observation does not exclude an interdependence of biotin and pantothenic acid with other vitamins in metabolism, since some physiological processes occur slowly and could not be discerned within so short an observation period. Although the physiologic functions of biotin and pantothenic acid are less well understood than those of other vitamins, it is probable that they act independently.

Thiamine, riboflavin, and niacin are generally considered oxidative catalysts and therefore to be closely related in metabolism and physiologic functions. The relative levels

desirable for each component in the diets of human beings for the optimum nutrition of either sex at various age levels are not known. The ratios of thiamine to riboflavin in the intakes of our experimental subjects were increased by the amounts of those vitamins included in the supplements. With two exceptions, the ratios for the daily urinary excretions showed corresponding changes from supplementation. Contrariwise, the milk secreted during the final 24 hours of supplementation contained greater amounts of riboflavin in relation to thiamine than did the mean daily secretions in milk for the control periods.

The food requirements of the nursing mother and her infant are not fully known, nor have the interrelationships among the vitamins been explored sufficiently to determine what proportions are most suitable for them. The maternal diet is known to influence the concentrations of nutriments in breast milk and thereby to change the nutrient quality of the food the baby consumes, but the levels and proportions of vitamins best suited for an infant are unknown, although studies by this laboratory have included investigations of the vitamin C in cord and infant blood and the urinary excretion of vitamin C, thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotin by newborn infants (Hamil, Munks, Moyer, Kaucher and Williams, '47; Hamil, Coryell, Roderuck, Kaucher, Moyer, Harris and Williams, '47).

Ceiling levels of concentration are provided by nature for certain vitamins in breast milk but not for others. The quantities of vitamins secreted in breast milk vary throughout the day, thus altering the quality of the milk consumed by the baby. The quantity and quality of milk also change with the progression of lactation. No doubt most breast-fed babies will thrive best if their mothers consume adequate mixed diets. In most instances healthy, successfully nursing mothers have satisfied babies. There is no indication that it is desirable for such mothers to supplement an adequate diet with synthetic vitamins. Until the nutritive requirements of mothers and babies are better known and our knowledge of the physi-

ology of lactation is enlarged, it seems unwise for the average healthy mother consuming a good diet to take synthetic vitamin preparations.

The ill health and undernutrition of either the nursing mother or her infant, however, are justification for taking vitamins under medical supervision. This and other studies (Pratt, Munks and Macy, '47; Lesher, Robinson, Brody, Williams and Macy, '48) of nursing mothers indicate that therapeutic doses of thiamine, riboflavin, niacin, vitamin A, and ascorbic acid are reflected in the composition of breast milk within a few days, the physiological response being of different intensity in different women and in the same subject at different times during lactation. Future investigation should determine the time required for the average healthy nursing mother accustomed to a good diet to make the physiological adjustments elicited by a changed level of intake and establish the physiological equilibrium characteristic of the individual. Such information is essential for complete understanding of the nutritive requirements of mothers and babies, and greater knowledge of body requirements would lead to elimination of some of the present nutritive hazards in childbearing and child rearing.

SUMMARY

Ten healthy multiparas in full milk production and successfully nursing their babies participated in one to 4 studies of the vitamin content of their milk and urine before and after ingestion of multi-vitamin supplements. Each of 18 studies consisted of control periods of 5 consecutive days followed by administration daily for 6 to 14 days of a supplement consisting of 750 mg ascorbic acid, 50,000 I.U. vitamin A, 12 mg thiamine chloride, 15 mg riboflavin, and 120 mg niacin in addition to an adequate diet. Results were obtained from analyses of 24-hour collections of food, breast milk, and urine for thiamine, riboflavin, niacin, pantothenic acid, and biotin.

The amounts of vitamins given did not stimulate milk production, since after supplementation equal numbers of studies showed increased and decreased volumes of milk.

The maternal organism responded rapidly to the B supplementations but the mothers demonstrated a wide range of physiologic response to vitamin supplementation, among individuals and for the same individual at different intervals in lactation.

The percentages of thiamine, riboflavin, and niacin intakes found in milk and urine increased with supplementation, though not consistently for all subjects nor in proportion to the increased intakes.

The amounts of pantothenic acid and biotin determined in milk and urine did not show the influence of the consumption of larger quantities of the other B vitamins.

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THE SYNTHESES AND INTERCONVERSIONS OF POLYUNSATURATED FATTY ACIDS BY THE LAYING HEN [1, 2]

RAYMOND REISER
WITH THE TECHNICAL ASSISTANCE OF BETTY GIBSON, MARY JOYCE CARR AND BEVERLY GENE LAMP
Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and Mechanical College System, College Station

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INTRODUCTION

Comparatively little information is available on the ability of animals other than the rat to synthesize polyunsaturated acids, or on the importance of these acids in their nutrition. Knowledge of the interconversions of polyunsaturated acids by the animal organism is just as meager and somewhat conflicting.

The high fat content of eggs makes the laying hen a particularly suitable animal for the study of fat metabolism. Poultry and egg fat are high in polyunsaturated acids, but it is not known whether the hen obtains these acids from its rations or by neogenesis. Neither is it known whether linoleic or linolenic acids are essential for the production or hatchability of eggs, though they appear to be essential for the growth of chicks (Reiser, '50a). It has been reported (Reiser, '50b) that the addition of linoleic (dienoic) acid in the form of cottonseed oil [3] to a fat-free synthetic hen ration resulted

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[3] Wesson.

in the deposition of dienoic, trienoic, tetraenoic and pentaenoic acids in the eggs. It has been found in the growing chick that the ingestion of dienoic acid results in the deposition of tetraenoic and pentaenoic acids (but not trienoic) and the ingestion of trienoic acid results in the deposition of hexaenoic (Reiser, '50c)

Studies with rats show sharp differences from these conclusions. Widmer and Holman ('50) did not find the production of pentaenoic acid from dienoic except in one unusual case. In contrast to the results of the present work, they did not find the production of "arachidonic" acid from linolenic acid. In a recent communication Holman ('51) has reversed his position on the latter point.

The previous study from this laboratory on the interconversion of fatty acids by the hen (Reiser, '50b) left a number of important questions unanswered. At the end of 14 weeks on the fat-free ration linoleic and linolenic acids were apparently still decreasing. It was thus uncertain whether these acids would eventually disappear or whether production would cease. The linoleic acid supplement was given in cottonseed oil, making any conclusions on the conversion to saturated or oleic acids impossible. It was also considered of interest to determine the transformations of linolenic (trienoic) acid. This acid, though common in some constituents of animal feeds, is seldom present in more than trace amounts in animal fats. The question of its disposition by saturation, desaturation, or preferential oxidation has never been answered.

The present work is an attempt to answer these questions and to confirm some previous observations.

PROCEDURES

General

As in the original studies, the hens used were 7-month-old White Leghorns just coming into production; they were placed in individual laying batteries and artifically insemi-

nated weekly. They were supplied with running water and fed daily.

Five groups of three hens each were placed on (1) the basal fat-free ration (2) 2% bayberry tallow, (3) 2% ethyl linoleate, (4) 2% tung oil and (5) 2% cod liver oil rations, respectively. The response to linolenate was determined by feeding 6 gm of trilinolenin in one experiment and 6 gm of linolenic acid in another in three 2-gm daily doses to hens that had been on a fat-free ration for 19 and 27 weeks, respectively.

Alternate eggs from each hen were analyzed and incubated. The eggs of any predetermined period, one or two weeks, were pooled and analyzed for polyunsaturated acids as described below. Groups 3, 4, and 5 were taken off experiment after 14 weeks. After 14 weeks the hens on the bayberry tallow ration were added to the group on the fat-free ration since their eggs showed the same changes. This group was then continued for 38 more weeks, or 52 weeks in all. Of the 6 hens, three died of unexplained causes in the 17th, 37th and 48th weeks, respectively. The last was analyzed. One was sacrificed for analysis after 25 weeks. Two survived and were in active egg production when the experiment was discontinued.

An effort was made to deplete the hens of their last stores of polyethenoid acids by fasting them during the 34th and 35th weeks.

Rations

Egg albumen was used as the protein source for the present investigation, instead of the soybean protein previously fed (Reiser, '50b). It was found that the latter contained spectrophotometrically measurable quantities of linoleic acid when extracted by acid hydroysis.[4] Egg albumen did not.

[4] The author has found that certain protein preparations, such as alcohol-extracted casein and soybean globulin, which apparently contain little or no fat when extracted in a Soxflet or Butt extractor, may contain as high as 2% fatty acid when hydrolyzed with hydrochloric acid. The method used is similar to that described in the "Official and Tentative Methods" of the Association of Official Agricultural Chemists for fat in cereal foods.

This necessitated certain changes in amino acid and biotin supplements. A liver extract[5] was used as the source of vitamin B_{12} and unknown factors. The ration, which contained no detectable amounts of polyunsaturated fatty acids, is given in table 1. When any lipid was included in the ration it replaced sucrose gram for gram.

TABLE 1

Basal fat-free ration

	Major constituents		
	%		
Sucrose	68	Choline	0.25
Glycine	1.7	Salts 5	5
Albumen (egg)	25	Inositol	0.125
	Water-soluble vitamins		
	mg/kg		*mg/kg*
Thiamine	6	Niacin	70
Ca-pantothenate	25	Folic acid	2.5
Pyridoxine	8	Biotin	5
	ml/kg		
Lederle's liver extract for B_{12}	6	Riboflavin	8
		p-Aminobenzoic acid	3
	Fat-soluble vitamins[1]		
	gm/kg		*mg/kg*
Mixed tocopherols	1	Carotene	33
	mg/kg		
Menadione	1.5	Vitamin D_3	0.08

[1] Mixed with equal weight of tween 20 and emulsified in water.

Preparation of test lipids

Ethyl linoleate was prepared from cottonseed oil by an alkaline interesterification procedure similar to that of Fuege and Gros ('49). The saturated and oleic acid esters were removed by the urea complex procedure (Schlenk and Holman, '50). The product contained 68.8% ethyl linoleate determined spectrophotometrically, but no more highly unsaturated acids.

[5] Lederle's.

The linolenic acid [6] had an iodine value of 272.5 (theory, 273.5).

Trilinolenin was obtained from Dr. R. T. Holman, of this laboratory. It was prepared from linolenic acid purified by the hexabromide procedure.

Analyses

The present method of analysis is much simpler than that previously described (Reiser, '50b) and can be used on one egg yolk. The yolks were extracted with chloroform and the isolated lipids were separated into acetone-soluble and -insoluble fractions. The polyethenoid acids of each were determined according to the method of Brice et al. ('45) without previous isolation of the fatty acids. Fatty acids were assumed to constitute 66.7% of the phospholipids. In calculating neutral fat, fatty acid cholesterol was considered as constituting 6.6% of the neutral fat of the yolk, and glycerol 10% of the glyceride fraction. (The cholesterol ester fatty acids, which need no correction for glycerol, would require an insignificantly small second correction.)

RESULTS

Fat-free ration

From table 2 it may be seen that the fatty acid composition of egg yolk lipids produced by hens on stock ration and by other hens after 14 weeks on a fat-free ration was approximately that previously reported (Reiser, '50b). When the fat-free diet was continued beyond 14 weeks, the more highly unsaturated acids continued to decrease. Hexaenoic acid disappeared even from the phospholipids in 17 weeks and the pentaenoic acid from the neutral fat in 18 weeks.

After 18 weeks, the fatty acid composition was somewhat erratic. In the neutral fat linoleic acid fluctuated between 1.1 and 1.5%, linolenic acid between 0.2 and 0.5%, and arachi-

[6] Obtained from the Hormel Foundation.

donic acid between 0.0 and 0.2%. In the phospholipids linoleic acid varied between 2 and 5%, linolenic between 1 and 3%, arachidonic between 2 and 3% and the $E^{1\%}_{1\ cm}$ of pentaenoic acid between 2 and 5. There was never any indication that hexaenoic acid returned to the neutral fat, though there were occasional indications of the presence of pentaenoic. Traces of hexaenoic acid were present in the phospholipids during some weeks. Since 4 and 5 double-bond acids are derived from the two double-bond acid (see below), one may expect them to make their apearance in the presence of the

TABLE 2

Fatty acid composition of yolk lipids of eggs laid by hens on fat-free ration

TIME ON RATION	SATURATED	OLEIC	DIENOIC 233 mμ	TRIENOIC 268 mμ	TETRAENOIC 316 mμ	PENTAENOIC 348 mμ	HEXAENOIC 376 mμ
weeks	%	%	%	%	%	$E^{1\%}_{1\ cm}$	$E^{1\%}_{1\ cm}$
			Neutral fat fatty acids				
0	31	53	14.4	0.18	1.43	2.51	1.12
5 and 6	28	69	2.6	0.19	0.33	0.72	0.42
14	31	67	1.3	0.22	0.23	0.43	0.00
18	27	70	1.9	0.31	0.00	0.00	0.00
52	30	68	1.2	0.42	0.12	0.00	0.00
			Phospholipid fatty acids				
0			20.1	0.0	9.1	9.20	3.79
5 and 6			4.7	1.1	4.4	6.09	2.55
17			4.1	3.7	2.7	3.38	0.00
52			2.7	2.3	2.2	3.50	0.00

latter. The same is true of hexaenoic acid derived from trienoic. However, there was no consistent change in any of the acids after 18 weeks.

Linolenic acid appears to increase as the other polyethenoid acids decrease, especially in the phospholipids.

It is interesting to note that the saturated acids remained constant, but that oleic replaced the lost polyunsaturated acids in the neutral fat.

The content and distribution of the egg lipids are independent of the ration. The total lipid, phospholipid, neutral

fat and cholesterol remain at 33, 11, 20.5 and 1.4%, respectively, as previously reported.

In table 3 are given the amounts of polyethenoid fatty acids in the hens at the beginning of the experiment and after 24 and 48 weeks on the fat-free ration. It is interesting that no hexaenoic acids were in the neutral fat of hens on stock rations, though there were appreciable quantities in their eggs. There was a rapid fall in dienoic acid during the first 24 weeks, from about 34 to about 6 gm. Trienoic and tetraenoic acid showed small losses. Hexaenoic acids disappeared from the neutral fat, but were unchanged in the phospholipids during this period.

During the next 24 weeks, and after a two-week fast, there was a further loss of 2 gm of dienoic acid and hexaenoic acid disappeared from the phospholipids.

A sample of mesentery fat was removed from a hen during the 31st week. It contained very small amounts of unsaturated acids, indicating that these acids remaining in the hen were in muscle and glandular tissues.

The two hens which survived the 52-week test were actively laying at its conclusion and the eggs were hatchable.

Bayberry ration

The compositions of the egg yolk fatty acids after 14 weeks on test rations are given in table 4. The addition of 2% bayberry tallow to the fat-free ration produced no effect on the fatty acid composition of the egg yolk lipids different from that of the fat-free ration.

Linoleate ration

Upon addition of linoleate, there is a gradual increase in dienoic and pentaenoic, but not in trienoic or hexaenoic acids. An apparent increase in tetraenoic acid may be due to an uncorrected spectral effect of the increased pentaenoic. It is now believed that dienoic is not converted to trienoic, although this had been concluded in the earlier study. As the dienoic acid content increased, the oleic acid content decreased.

TABLE 3

Fatty acid composition of hen lipids on stock and fat-free rations

TISSUE	RATION	TIME ON RATION	DIENOIC		TRIENOIC		TETRAENOIC		PENTAENOIC		HEXAENOIC	
			P [1]	NF [1]	P	NF	P	NF	P	NF	P	NF
		weeks	%	%	%	%	%	%	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$
Total	Stock	0	29.8	19.9	0.33	0.40	4.00	0.46	6.83	0.71	2.12	0.0
animal			33.6 gm		0.6 gm		1.1 gm					
Total	Fat-free	24	8.74	7.4	1.03	0.18	3.90	0.40	5.40	0.0	2.26	0.0
animal			6.2 gm		0.2 gm		0.6 gm					
Total [2]	Fat-free	48	5.43	3.56	0.29	4.13	3.71	0.17	5.20	0.0	0.0	0.0
animal			4.1 gm		0.4 gm		0.6 gm					
Mesentery	Fat-free	31	1.77		0.20		0.08		0.0		0.0	

[1] P = phospholipid; NF = neutral fat.

[2] Fasted during 34th and 35th weeks.

Tung oil ration

The saturated and oleic acid contents of the egg yolk acids could not be calculated because of the presence of the conjugated eleostearic acid. The increase in all polyunsaturated acids studied indicates a remarkable lability of conjugated trienoic acid and a mechanism for its interconversion, both to lesser and to more highly unsaturated acids. A study of the metabolism of conjugated fatty acids is in press (Reiser, '51).

TABLE 4

Compositions of fatty acids of egg yolk lipids after 14 weeks on test rations

RATION	SATURATED	OLEIC	DIENOIC	TRIENOIC	TETRAENOIC	PENTAENOIC	HEXAENOIC
	%	%	%	%	%	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$
			Neutral fat fatty acids				
Stock	31	53	14.4	0.18	1.43	2.61	1.12
Fat-free	31	67	1.3	0.22	0.23	0.39	0.00
Bayberry	32	66	1.6	0.24	0.22	0.40	0.00
Linoleate	31	53	14.7	0.00	0.90	0.91	0.00
Tung oil [1]			5.6	2.75	1.77	2.19	0.62
Cod liver oil	31	65	2.3	0.00	1.30	4.45	1.50
			Phospholipid fatty acids				
Stock			20.1	0.00	9.10	9.20	3.79
Fat-free			4.4	2.99	3.55	3.50	1.24
Bayberry			3.7	2.15	4.62	3.60	1.15
Linoleate			12.6	0.00	11.6	7.08	1.70
Tung oil			8.3	0.00	8.42	15.7	7.31
Cod liver oil			8.9	0.00	13.7	31.4	7.55

[1] The hens were fed tung oil for only 11 weeks.

Cod liver oil ration

Cod liver oil was used as a source of the higher unsaturated fatty acids, free of linoleic and, probably, linolenic. It was hoped that by its use some hint might be gleaned of the production of the less unsaturated from the more highly unsaturated acids. Unfortunately, due to the impossibility of determining the spectral effects of pentaenoic and hexaenoic acids on the lower members of the series, the results given in table 4 for cod liver oil are equivocal. The calcu-

lated increase in the percentage of linoleic acid in the eggs of the hens fed cod liver oil, as compared to the increase in those of the fat-free group, could have been due to the much larger quantities of pentaenoic and hexaenoic acid in the former.

TABLE 5

Fatty acid changes in the egg yolk neutral fat fatty acids of hens fed 6 gm of trilinolenin or linolenic acid after having been on a fat-free ration

EGGS AFTER TEST MEAL	DIENOIC ACID 233 mμ		TRIENOIC ACID 268 mμ		TETRAENOIC ACID 310 mμ		PENTAENOIC ACID 348 mμ		HEXAENOIC ACID 376 mμ	
	Ester [1]	Acid [2]	Ester	Acid	Ester	Acid	Ester	Acid	Ester	Acid
	%	%	%	%	%	%	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$
Fat-free control	1.44	1.10	0.19	0.21	0.14	0.11	0.14	0.39 [3]	0.04 [3]	0.26 [3]
0	1.32	1.22	0.14	0.19	0.13	0.16	0.14	0.31 [3]	0.04 [3]	0.19
1	1.42	1.45	*0.27*	*0.27*	0.10	0.14	*0.18*	*0.49*	0.05 [3]	*0.29*
2	*1.88*	*1.67*	1.13	0.35	*0.26*	*0.28*	0.54	0.62	*0.17*	0.33
3	1.72	2.22	1.69	1.03	0.47	0.57	1.06	0.78	0.39	*0.32*
4	2.16	2.35	1.60	1.10	0.56	0.76	1.36	0.93	0.44	0.31
5	2.23	2.46	0.56	0.75	0.60	0.67	1.06	0.99	0.39	0.40
6	2.52	2.82	0.36	0.94	0.46	0.75	1.34	1.30	0.59	0.55
7	2.42	1.72	0.07	0.47	0.47	0.16	1.11	1.02	0.51	0.47
8	2.28	2.51	0.17	0.36	0.19	0.42	0.43	1.17	0.00	0.64
9	1.38	2.49	0.29	0.11	0.08	0.42	0.27	0.58	0.00	0.26

[1] Trilinolenin ingestion.

[2] Linolenic acid ingestion.

[3] No maximum. The presence of the acid questionable.

Linolenic acid conversions

In order to study the effects of non-conjugated trienoic acid, 6 gm of trilinolenin and 6 gm of linolenic acid were fed in three 2-gm daily doses in two different tests to hens which had been on the fat-free ration for 19 and 27 weeks, respectively. The results given in tables 4 and 5 show the same lability of this trienoic acid as was found with eleostearic. The "fat-free control" is the composition of the last egg laid by the hen before the test meal. The "O" egg is that laid the day the first dose of the test meal was given.

The trienoic acid and pentaenoic acid content began to increase in the neutral fat fraction in the first egg laid after the first dose of the test meal. The other acids showed first increases in the second egg. The eggs in which the acids reach the maximum levels do not show the same clear relationship, although the trienoic acid does reach the maximum level first, in the third and 4th egg, followed by the other acids in the 6th egg. The maximum level of tetraenoic acid may have been reached in an earlier egg.

In table 6 are given the fatty acid contents of the phospholipids. The trienoic acid content, as given, is not logical. The calculated value is probably in error due to the large amounts of pentaenoic and hexaenoic acids present. This will be discussed below. The tetraenoic acids were the first to show a clear increase, this occurring in the second egg, followed by increases in the other acids in the third egg. An exception is the tardy appearance of dienoic acid when the ester was fed.

DISCUSSION

There is some error in all the quantitative values given in this paper in any case in which pentaenoic or hexaenoic acid is present in significant amounts. This error is inherent in all spectrophotometric determinations of fatty acid mixtures containing acids of more than 4 double bonds, and is due to lack of knowledge of the spectral effects of these acids on the less highly unsaturated acids. The values for uncorrected tetraenoic acid are too high. The values for the other acids may be high or low, depending on whether the uncorrected spectral effect of the 5 and 6 double-bond acids is greater or less than the too-great correction for the tetraenoic acid. It may be this error which produces the illogical trienoic acid content of phospholipid fatty acids given in table 6. This table shows a decrease in trienoic acid after its ingestion, as the more highly unsaturated acids increase. It is quite possible that linolenic acid does decrease with an increase in the more highly unsaturated acids, since it is clear that

it increases as they decrease on a fat-free regimen. However, it does not seem logical that the acid should decrease as a result of its ingestion.

It is obvious from these studies that the hen does not itself produce from non-fat precursors the large amounts of polyunsaturated fatty acids considered typical of its tissues and eggs. After a full year the yolk neutral fat of the eggs of hens on the fat-free ration contained only about 1% linolenic

TABLE 6

Fatty acid changes in the egg yolk phospholipid fatty acids of hens fed 6 gm of trilinolenin and linolenic acid after having been on a fat-free ration

EGGS AFTER TEST MEAL	DIENOIC ACID 233 mμ		TRIENOIC ACID 268 mμ		TETRAENOIC ACID 310 mμ		PENTAENOIC ACID 348 mμ		HEXAENOIC ACID 376 mμ	
	Ester [1]	Acid [2]	Ester	Acid	Ester	Acid	Ester	Acid	Ester	Acid
	%	%	%	%	%	%	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$
Fat-free control	5.36	2.38	1.56	2.64	2.22	2.29	4.56	2.57	1.85	1.07
0	3.35	3.66	*2.45*	2.41	2.33	2.37	3.95	2.52	1.57	1.20
1	4.02	4.51	2.08	2.22	2.40	2.83	3.03	2.96	0.87	1.14
2	4.67	3.70	2.11	2.06	*3.21*	*3.93*	4.88	4.98	1.42	1.97
3	*6.20*	1.57	1.05	2.21	4.74	5.18	*9.06*	*7.22*	*3.03*	*3.00*
4	6.49	3.84	0.00	*3.46*	4.10	6.87	9.11	8.22	3.21	2.72
5	5.13	*8.26*	0.00	0.00	5.32	7.08	9.35	10.50	3.46	3.76
6	5.83	10.45	0.33	0.00	4.44	7.41	6.31	9.18	3.58	3.16
7	2.25	8.96	0.00	0.00	3.10	7.52	7.92	8.36	3.74	2.94
8	3.92	8.79	0.20	0.00	3.66	7.44	3.46	7.70	1.36	2.75
9	3.97	9.49	0.95	0.00	2.44	6.55	4.09	7.48	1.60	3.18

[1] Trilinolenin ingestion.

[2] Linolenic acid ingestion.

acid, as compared to 14.4% in the eggs of hens on a stock ration.

Linolenic acid in the egg approaches a minimum value of between 1 and 1.5% in the neutral fat fatty acids and between 2 and 5% in the phospholipid acids. Since these were not supplied in the ration, and could no longer be supplied from the tissue fats, as is indicated by the small amounts remaining in the hen, one must conclude that they

were supplied by synthesis. There were no polyethenoid acids in the droppings, which probably eliminated the possibility of intestinal synthesis.

Since, as demonstrated by the ingestion of linoleic acid, dienoic acid is converted to tetraenoic and pentaenoic acid by the hen, the neogenesis of linoleic acid can explain the presence of the other two.

The increase in linolenic acid, in both neutral fat and phospholipid, as the other polyunsaturated acids decreased, has no obvious explanation. The absence of, or very low, values in eggs from birds on stock ration or after the ingestion of linoleate, tung oil and cod liver oil (table 4) may be artifacts due to too large corrections in the presence of pentaenoic and hexaenoic acids, as was pointed out above. However, the high values of phospholipid trienoic acid on a fat-free ration must be factual. They cannot be mathematically accounted for by the low values of 5 and 6 double bond acids.

The interesting questions relating to the interconversions of the polyunsaturated acids are, in part, answered. No polyunsaturated fatty acids are formed from saturated acids. The interconversions of the linoleic acid of the ration are fairly clear. It is evident (table 2) that pentaenoic acid, reduced to very low levels in the fat-free ration, is present in more significant quantities when dienoic acid is included. The present author has previously shown this to be true in the cases of eggs (Reiser, '50b) and of growing chicks (Reiser, '50c), in which latter case pentaenoic acid was absent on the fat-free ration but made its appearance when linoleic acid was added. Widmer and Holman ('50) did not find significant production of pentaenoic acid in the rat except in one unusual case.

The possible increase of tetraenoic acid when linoleate was fed (table 4) is equivocal, since its apparent increase may have been due to the uncorrectable increase of pentaenoic acid.

The production of trienoic acid from dienoic acid previously reported (Reiser, '50b) probably does not take place.

The earlier conclusion was based on studies of the extinction coefficients. When the percentages of the acids are calculated, the increases in trienoic acid disappear. This has also been shown to be the case in growing chicks (Reiser, '50c).

Trienoic acid is rapidly converted to all the other polyunsaturated acids considered. Whether fed as the conjugated eleostearic acid (table 2) or as linolenic acid (tables 5 and 6), it produces an unequivocal increase in dienoic, tetraenoic, pentaenoic and hexaenoic acids. This rapid conversion of trienoic acid explains the often observed decrease of this acid in animal lipids even after ingestion in considerable quantities (Shorland, '50; Beadle et al., '48). The question of whether linolenic acid is preferentially oxidized (Bloor, '43) or converted to other acids (Ellis and Isbell, '26) appears to be answered in favor of the latter in the case of the laying hen. The finding of the conversion of trienoic acid to the other polyunsaturated acids by the laying hen is in contrast to the nutrition studies of the "essential" fatty acids in the rat (Burr, '42; Greenberg, Deuel and Brown, '50; Greenberg, Calbert, Savage and Deuel, '50), in which it has been demonstrated that linolenic and linoleic acids have different roles. These differences in the interconversion of trienoic acid, and the conversion of dienoic to pentaenoic acid in the hen but not the rat, show very definite class differences in fatty acid metabolism.

The results of feeding linolenic acid to a hen that had been on the fat-free ration confirm the observations after tung oil ingestion that there is a mechanism present in the hen which rapidly converts trienoic acid to lower and more highly unsaturated acids. It was hoped that this procedure might disclose the sequence of these interconversions. The results indicate that if there is a sequence it takes place very rapidly, the only indications being that pentaenoic appears first in the neutral fat and tetraenoic first in the phospholipids.

An interesting observation is the relatively constant level of saturated acids in the neutral fat. Changes in the polyunsaturated acids, which are large in the case of linoleic

acid, are reflected in the oleic acid moiety. By feeding very large amounts of liquid oils (28%) reductions in the percentage of saturated acids in egg fat have been obtained (Cruickshank, '34).

SUMMARY AND CONCLUSIONS

White Leghorn hens in full egg production were placed on a sucrose, egg albumen basal ration containing no polyunsaturated acids or with 2% bayberry tallow, linoleate, tung oil, or cod liver oil added. In two experiments a hen on the fat-free ration was given pure trilinolenin and linolenic acid during 19 and 27 weeks, respectively. Polyethenoid fatty acids were determined in the neutral fat and phospholipids of subsequent eggs. It was found:

1. Hexaenoic fatty acid completely disappears from the neutral fat and phospholipids of the eggs of hens on a rigid fat-free diet. Pentaenoic acid disappears from the neutral fat but linoleic acid reaches minimum values in both neutral fat and phospholipids.

2. Laying hens on a fat-free ration can synthesize the minimum amount of polyethenoid acids which are maintained in the neutral fat and phospholipid of egg yolk. Hatchability and production do not appear to be affected.

3. Oleic acid replaces polyunsaturated fatty acids in the neutral fat of eggs produced by hens on a fat-free ration, the saturated acids remaining comparatively constant.

4. On the fat-free ration, trienoic acid makes its appearance as the other polyunsaturated acids decrease.

5. The addition of bayberry tallow to the ration had no stimulating effect on the deposition of polyethenoic acids.

6. The addition of dienoic acid to the ration resulted in an increase in dienoic and pentaenoic acids and a possible increase in tetraenoic. Trienoic and hexaenoic acids did not increase.

7. The addition of trienoic acids (eleostearic and linolenic) resulted in increases in all polyunsaturated acids having from two to 6 double bonds.

8. The total lipid, phospholipid, neutral fat or cholesterol composition of egg yolk is unaffected by the kind or amount of fatty acids in the ration.

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The author is happy to have this opportunity again to express his appreciation to Dr. John H. Quisenberry, Dr. J. R. Couch, and Mr. H. L. German, of the Poultry Department of the Texas Agricultural Experiment Station, for their unstinted cooperaton, so necessary for the successful pursuit of these investigations.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT [1, 2]

II. INVESTIGATION OF THE NUTRITIOUSNESS OF THE PRODUCTS OF THERMAL POLYMERIZATION OF LINSEED OIL

EARLE W. CRAMPTON, R. H. COMMON, FLORENCE A. FARMER, F. MARION BERRYHILL AND L. WISEBLATT

Departments of Nutrition and Chemistry, Macdonald College, McGill University, Province of Quebec, Canada

FOUR FIGURES

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INTRODUCTION

Heating unsaturated oils to polymerization temperatures has been advocated as a practical method of reducing or eliminating the flavor reversion of shortening produced from such oils (Privett et al., '45). However, if oils treated in this manner are included in the diets of rats, then the rate of gain, the feed intake and the feed efficiency are decreased. These effects may be due to the action of some toxic material (Crampton et al., '51).

Of the oils which have been tested in this laboratory, linseed oil has proved to be the one most susceptible to nutritive damage by heating. Accordingly, linseed oil was selected for further studies on the nature of the material responsible for the toxic effects. The results of these experiments are reported in the present paper.

[1] Contributon from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series No. 280.

[2] Issued as paper 263, Canadian Committee on Food Preservation.

Unsaturated fats yield dimeric, trimeric and higher polymeric glycerides when heated at 275°C. in the absence of oxygen. It is rather generally accepted that polymerization does not proceed to any appreciable extent beyond dimerization so far as the fatty acid radicals themselves are concerned (Privett et al., '47). The extent to which such dimerization of the fatty acid radicals may result in the presence of intrapolymeric glycerides remains controversial. Indeed, Bernstein ('48) has dismissed the possibility on theoretical grounds. Adams and Powers ('44), on the other hand, produced considerable experimental evidence in support of the view that such intrapolymers are formed in the early stages of polymerization, and that they may in turn give rise to dimeric and higher polymeric glycerides by transesterification. Experimental evidence obtained in our laboratories supports the contention that thermally polymerized linseed oil may contain considerable amounts of intrapolymeric material (see appendix, table 1). Kass ('47) studied the thermal polymerization of eleostearates, and, as well as observing normal polymerization products, described side reactions leading to production of free fatty acids, aldehdyes, hydrocarbons and acids and esters of reduced molecular weight. He found that some of the latter fragments also polymerize, and produced evidence of cyclization leading to the production of cyclic aliphatic monomers and mono- and polycyclic aromatic compounds. We have essayed to indicate in plate 1 the nature of the substances which are thought to be the main products of thermal polymerization.

At the outset we suspected that polymerized triglycerides might be mainly responsible for the lower nutritive value of heated linseed oil. Consequently we sought to obtain for feeding tests a fraction which would consist essentially of non-polymeric glycerides. Privett et al. ('47) have described the preparation from heated linseed oil of an acetone-soluble segregate which, they reported, was free from "dibasic acids and was, therefore, non-polymeric in nature." Bernstein ('48) has described the use of propanol for the segre-

gation of monomeric from higher polymeric glycerides, such monomeric fractions being regarded by him as free from dimeric acids. In view of these reports, segregates were prepared by the use of acetone and also by the use of propanol. Our chemical data (see appendix, table 2) indicate that propanol was more effective than acetone for recovery of a non-polymeric glyceride fraction, thus supporting the findings of Bernstein in this respect. We have shown, however, that the acetone-soluble segregate of polymerized linseed oil appears to contain appreciable amounts of intrapolymeric material (see appendix, table 1). The propanol segregate does not contain dimeric or higher polymeric glycerides, but we have not examined this fraction for the presence of intrapolymers. Presumably the principal polymerized material, if any, in the propanol segregate is intrapolymeric. The acetone-soluble fraction is probably composed mainly of the molecular types indicated in plate 1, figure 2, while the propanol-soluble fraction is probably composed of those indicated in plate 1, figure 3.

It was also thought possible that the dimeric fatty acid radicals themselves might be implicated in the nutritional defects of polymerized linseed oil. Since solvent segregations offered little promise for the separation of true monomeric glycerides from intrapolymeric glycerides, it was decided to esterify the heated oil and then to separate monomeric esters from dimeric esters by distillation under vacuum. The esterification yielded the fractions indicated in plate 1, figure 4, and the succeeding distillation under vacuum achieved an essentially complete separation of polymeric material (see appendix, table 3). The distillate consisted of monomeric esters, but it may possibly have included some cyclic monomeric material. The undistillable residue consisted essentially of esters of dimeric acids, together with such sterols as were present and presumably some non-volatile products of thermal decomposition which were soluble in acetone.

EXPERIMENTAL PROCEDURE

The materials prepared for feeding comprised fractions of heated linseed oil composed largely of (a) monomeric esters, and (b) dimeric esters, as well as several fractions which contained various mixtures of monomeric and dimeric glycerides and of intrapolymers. In addition to examining biologically the fractions of the oil segregated by solvents (acetone and propanol) and by distillation of the esters of the fatty acids, crude and refined heated oils were compared to discover some indication as to whether or not the non-glyceride constituents of the oil were concerned in growth inhibition.

Polymerization and acetone segregation were carried out by the technique of Privett, McFarlane and Gass ('47). The polymerizations were based on a treatment of 12 hours at 275°C. in an all-glass apparatus while passing through it a current of carbon dioxide. A sufficient flow of carbon dioxide was maintained to keep the oil surging vigorously; attention to this detail resulted in products of excellent color and blandness and with remarkably low acid values (of the order of 0.6 to 1.0% F.F.A.). Propanol segregations were performed according to the directions of Bernstein ('48). Esterifications were carried out by interesterification in the presence of the appropriate anhydrous alcohol, using sodium hydroxide as catalyst; the oil or fraction was alkali-refined to remove F.F.A. prior to esterification. Esters were distilled at about 0.5 mm Hg absolute pressure (see appendix).

The several fractions of linseed oil, indicated in table 1, were fed to albino rats as the source of dietary fat for 28-day periods. The animals ranged from 21 to 28 days of age at the outset of the tests.

The basal diets had the composition described in table 2. Diets were baked for 20 minutes at 375°F. and the resulting biscuit granulated and air-dried for 24 hours. Vitamin B supplements, in the case of diet II; or yeast, in the case of diet III, were then added. Weekly doses of cod liver oil were

administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

FEEDING TRIAL RESULTS

Table 1 is a summary of the weight gains and feed intakes of animals fed the various fractions of linseed oil. It is recognized that negative gains are unsatisfactory as quantitative measures of the nutritional values of rations, nor are

TABLE 1

Summary of average daily gains and average feed intakes of animals fed diets containing 20%[1] of various fractions of linseed oil

TEST NO.	OIL FRACTION	TREATMENT AT 275°C.	PRINCIPAL COMPONENTS OF FRACTION	NO. ANIMALS	AVE. DAILY GAINS	AVE. DAILY FEED INTAKE
		hrs.			*gm*	*gm*
1. A	Whole	none	Monomeric glycerides	5	3.0	6.8
B	Whole	12	Monomeric glycerides Intrapolymers Dimeric and polymeric glycerides	5	−3.0	4.6
C	Acetone-soluble	12	Monomeric glycerides Intrapolymers Dimeric glycerides	5	−3.0	5.3
2. A	Acetone-soluble, alkali-refined	12	Monomeric glycerides Intrapolymers Dimeric glycerides	6	−2.1	4.5
B	Acetone-soluble, crude	12	Monomeric glycerides Intrapolymers Dimeric glycerides Non-glyceride constituents	6	−0.7	3.5
3. A	Whole	none	Monomeric glycerides	10	3.8	10.0
B	Acetone-soluble	12	Monomeric glycerides Intrapolymers Dimeric glycerides	10	−1.4	6.0
C	Propanol-soluble	12	Monomeric glycerides Intrapolymers	10	0.2	5.5
4. A	Whole	none	Monomeric glycerides	8	3.4	9.7
B	Whole ethyl esters	none	Monomeric esters	8	2.3	7.5
C	Acetone-soluble ethyl esters	12	Monomeric and dimeric	8	0.8	5.8
D	Distillate of 4C	12	Monomeric esters	8	0.7	4.5
E	Residue of 4C	12	Dimeric esters	8	[2]	3.5

[1] Excepting test 2, where 36% fat was used.

[2] No gain could be calculated, as 6 out of 8 animals died within the first week. The remaining two survived three and 4 weeks, respectively.

statistical analyses of weight losses justified. In these trials such data are nevertheless indicative of the differences in nutritive quality of the diets studied. Where weight losses were encountered (tests 1, 2, and 3), the diets may be judged unwholesome without the benefit of statistics. However, it was possible to make an analysis of the variance of both gain and feed intake in test 4. Differences significant at the 5% point were apparent between A and B, B and C, and B and D. C and D were not different.

TABLE 2

Composition of diets

INGREDIENTS	DIET I (test 2)	DIET II (test 1)	DIET III (tests 3 and 4)
White flour (%)	26.5	47.0	44.0
Casein (%)	15.0	11.5	11.5
Milk powder (%)	20.0	19.0	19.0
Linseed oil (%)	36.0	20.0	20.0
Bone meal (%)	2.0	2.0	2.0
Salt (%)	0.5	0.5	0.5
Yeast (%)	..	..	3.0
Thiamine	[1]	10 p.p.m.	..
Niacin	[1]	230 p.p.m.	..
Riboflavin	[1]	5 p.p.m.	..

[1] Where diet I was fed (test 2), thiamine, niacin and riboflavin were administered orally to provide daily intakes of 0.1 mg, 2.3 mg, and 0.5 mg, respectively, per rat.

It is realized that purified esters are of uncertain feeding value because of their susceptibility to oxidative changes (Quackenbush et al., '42). For this reason, the effects on rat growth of esters vs. the triglycerides were compared. Animals fed esterified unheated oil gained significantly less weight than animals fed the unheated triglyceride; the feed intake of ester-containing diets decreased progressively during storage at room temperatures, indicating decreasing palatability of those diets. However, the utilization of feed for producing weight gain was not significantly different between the ester- and triglyceride-containing diets, nor could any clinical in-

dication of the toxicity of the esters be found. Esters of heated linseed oil showed the same adverse clinical effects as did the heated triglycerides, as will be seen in table 1.

DISCUSSION

The data shown in table 1 confirm that 12 hours' heating at 275°C. impairs the nutritive value of linseed oil.

A comparison of heated, whole oil with the acetone-soluble fraction (test 1) shows both of them to be equally incapable of supporting normal growth in rats. One may conclude, therefore, that acetone does not segregate toxic from nontoxic material.

There was no difference between the crude and refined oils (test 2), suggesting that the deleterious material in polymerized oil is derived from the glyceride component.

The propanol-soluble fraction was not significantly better nutritionally than the acetone-soluble fraction (test 3). It thus follows that the glyceride polymerization is not responsible for growth inhibition by heated linseed oil. Since neither acetone nor propanol is effective in segregating out the toxic material in heated linseed oil, one naturally turns to a consideration of the intrapolymers as the offending material. An interpolymer is a triglyceride within which two fatty acid chains are dimerized, and one might suspect the dimeric fatty acid structure of being the deleterious factor in heated oil. It is the only quantitatively important component of the propanol-soluble fraction of heated oil that is not present in unheated oil. How far this surmise is correct is evident from test 4, in which the "dimeric" fraction separated from the ethyl esters of the acetone segregate of polymerized oil was fed.

This undistillable "dimeric" fraction, which may also contain minor amounts of non-fatty material, proved lethal to rats in approximately 5 days.

The animals fed the distillable fraction (i.e., monomeric esters), although they did not grow, were thrifty in appear-

ance and excreted well-formed feces. Those fed the undistillable residue had oily, matted coats and excreted dark, sticky feces. This condition was also noted, but to a lesser degree, with the rats fed the mixed monomeric plus dimeric esters, and had also been recorded in previous tests in which the acetone-soluble fraction of heated oil had been fed. It appears possible, therefore, that the explanation of the failure to gain in weight on a diet containing the monomeric esters may be the absence of an essential unsaturated fatty acid, presumably because these more reactive acids had become polymerized or isomerized during heating. Thus dimerization may not only remove essential fatty acids, but produce toxic radicals as well. The strong adverse reaction of the rats to the dimeric esters may be the consequence of the absence of a needed growth factor plus the presence of a toxic agent.

CONCLUSIONS

On the basis of these results one might tentatively conclude that the primary cause of the lowered nutritive value of diets containing thermally polymerized linseed oil is the presence of one or more dimeric fatty acid radicals which are in some way inimical to the well-being of the animals. Their deleterious effects could well be aggravated since they are produced at the expense of unsaturated fatty acids, some of which may be those essential to the animal for growth.

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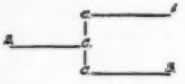
Thanks are also due to Mr. D. Mills, Department of Chemistry, Macdonald College, for his assistance in the preparation and analyses of the oils.

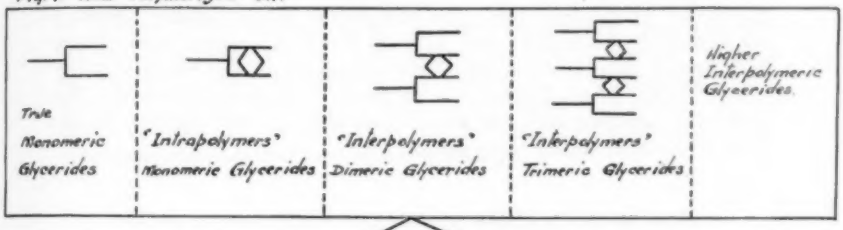
DIAGRAMMATIC REPRESENTATION OF THE MAIN CONSTITUENTS OF LINSEED OIL FOLLOWING THERMAL POLYMERIZATION, SOLVENT SEGREGATION AND ESTERIFICATION.



Initial triglyceride showing the position of the three fatty acid chains attached to the glycerol molecule.

Heating at 275°c.

Fig. 1. Heat Polymerized Oil.



Acetone Segregation

Propanol Segregation

Fig. 2. Acetone Soluble Segregate.

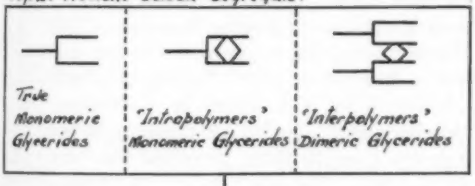
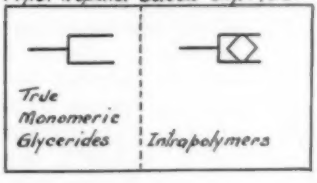
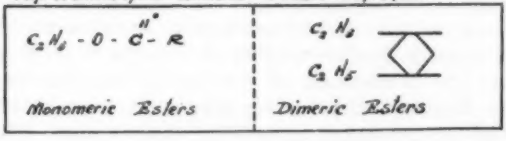


Fig. 3. Propanol Soluble Segregate.



Interesterification with Anhydrous Ethanol

Fig. 4. Esterified Acetone Soluble Segregate.



APPENDIX

Distillation of ethyl esters of the acetone-soluble fraction from polymerized linseed oil

The distillation of the esters was carried out using simple distilling bulbs and receivers and a glycerol bath. The distilling bulb was filled with clean glass wool to reduce bumping and frothing. Distillation temperature limits were established on esters of alkali-refined whole linseed oil, and these were observed closely in all distillations of esters from polymerized oils. The distillation pressures were of the order of 0.5 mm Hg. The distillable esters usually distilled completely between 155° and 175°C. When the vapor temperature had passed its maximum, the bath temperature was taken steadily up to 240°C. If no further rise in vapor temperature occurred, the bath was removed and the residue allowed to cool rapidly in the bulb without breaking the vacuum.

Demonstration of the presence of intrapolymers in the acetone-soluble fraction of polymerized linseed oil

A series of linseed oils were polymerized in a current of CO_2 for varying periods at 275°C. The methyl esters of their acetone-soluble segregates were prepared by interesterification. Cryoscopic determinations of mean molecular weight were carried out on the acetone-soluble fractions themselves and on the methyl ester fractions. The cyclohexane (British Drug Houses) used as cryoscopic solvent was carefully purified by successive treatment with fuming sulfuric acid and barium hydroxide, followed by distillation in a Stedman column. The purified solvent had M.P. 6.4°C., refractive index (25°C.) = 1.4237, values which are close to those recorded for very pure cyclohexane (Glasgow et al., '46). The observed mean molecular weights were corrected for the presence of free fatty acids by the method of Bernstein ('48), which assigns to the free fatty acids a dimeric average mean molecular weight of 558; i.e., assumes that association of the free fatty acids is practically complete. From the cryoscopic mean molecular weights of the acetone-soluble fraction, and assuming the presence of monomeric and dimeric glycerides of theoretical mean molecular weights of 878 and 1756, it was possible to calculate the percentages of monomeric and dimeric glycerides. From these figures, and assuming that polymerization of fatty acid radicals had not proceeded beyond the dimeric stage (Privett et al., '47), a theoretical yield of monomeric and dimeric esters could be calculated. The difference between these values and the observed

values for monomeric and dimeric esters obtained by distillation may be reasonably ascribed to the presence of intrapolymers, which would behave as monomeric glycerides in the mean molecular weight determinations. The percentage differences can be converted to a triglyceride basis by multiplying by 3/2. The results are set out in appendix table 1, and provide support for the belief that the acetone-soluble fractions of heated linseed oil contain considerable amounts of dimeric fatty acid in the form of intrapolymers.

Even after 13 hours of heating, the acetone-soluble fraction contained approximately 30% of its total fatty acids in the form of dimeric acids, but only approximately 12% could be accounted for as dimeric glyceride and some 19% of dimeric acid was probably present as intrapolymers. The amount of intrapolymers (soluble in acetone) would be expected to decline as polymerization progresses. It seems quite clear from these results that solvent segregation methods are unlikely to be effective in differentiating between intrapolymers and true monomeric glycerides.

Superiority of propanol over acetone in segregating a non-polymeric glyceride fraction from thermally polymerized linseed oil

The analytical data in table 2 show that both the viscosity and the refractive index of the propanol-soluble fraction were lower than those of the acetone-soluble fraction. The data confirm that propanol is the more effective solvent in segregating a non-polymeric fraction from thermally polymerized linseed oil.

Effectiveness of vacuum distillation in separating monomeric from dimeric ethyl esters of the acetone-soluble segregate of polymerized linseed oil

The analytical data presented in appendix table 3 relate to two samples of methyl esters prepared from acetone-soluble fractions of heated linseed oil. The data show that the "distillable" fraction consisted essentially of methyl esters of monomeric fatty acids, the mean molecular weights being 296 and 295. The "non-distillable" fraction consisted essentially of methyl esters of dimeric fatty acids, the mean molecular weights being 587 and 596.

The fact that refractive indices and iodine values for the recombined samples were practically the same as the values for the charge before distillation is evidence that the heating during distillation had little, if any, effect on the constitution of the esters.

APPENDIX TABLE 1

Calculation of the intrapolymer content of the acetone-soluble fractions of thermally polymerized linseed oil

HOURS HEATED	MEAN MOLECULAR WEIGHT OF GLYCERIDES OF HEATED OIL BY CRYOSCOPY	CALC. % DIMERIC GLYCERIDE	CALC. % DIMERIC METHYL ESTERS	OBSERVED % DIMERIC METHYL ESTER BY DISTILLATION	DIFFERENCE TO BE ASCRIBED TO INTRA-POLYMERS
5	1,010	36.1	8.7	21.7	13.0
9	1,030	29.6	9.7	23.1	13.2
13	1,060	34.4	11.5	30.7	19.2
17	970	19.0	6.3	14.8	8.5

APPENDIX TABLE 2

Analyses of acetone and propanol fractions from heated alkali-refined linseed oil

FRACTION	YIELD	REF. IND. 25°C.	VISCOSITY AT 250°C. CP.	IODINE VALUE
	%			
1. Unheated, recovered from acetone	..	1.4789	50	183
2. Heated, acetone-soluble	45	1.4811	180	134
3. Heated, propanol-soluble	28	1.4791	127	135
4. Heated, propanol-insoluble	72	1.4851	890	121

APPENDIX TABLE 3

Properties of methyl esters and fractions obtained in distillation

SAMPLE	FRACTION	PER CENT YIELD	MEAN MOLECULAR WEIGHT	REF. IND. AT 250°C.	IODINE VALUE
1	Charge (acetone-soluble)	...	334	1.4648	128.0
	Distillate	82.0	296	1.4600	137.2
	Residue	17.5	587	1.4896	105.5
	Recombined sample [1]	...	...	1.4647	127.6
2	Charge (acetone-soluble)	...	336	1.4647	129.0
	Distillate	81.9	295	1.4600	135.6
	Residue	17.6	598	1.4904	102.8
	Recombined sample [1]	...	...	1.4647	128.4

[1] Distillate and residue blended in the exact residue of their yields, to approximate original sample.

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